OVARIAN SYNCHRONIZATION AND SUPERSTIMULATION IN WOOD BISON (*Bison bison athabascae*)

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in partial fulfilment of the requirements for the

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Saskatoon

By

Jesus Manuel Palomino

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ABSTRACT

For this thesis our objectives were to establish an efficient method of ovarian synchronization and superstimulation in bison, and determine the effects of gonadotropin treatments on oocyte collection efficiency and quality in bison. In the first study we conducted two experiments to develop an efficient protocol for synchronization of follicular wave emergence during the anovulatory season. In Experiment 1, we compared the synchronizing effect of follicular ablation (n = 9) and treatment with 2 mg estradiol (E-) 17β in oil (n = 10), while in Experiment 2, we compared follicular ablation (n = 9) and treatment with 2 mg E-17β + 100 mg progesterone (P4; n = 10). Results showed that the degree of synchrony did not differ between ablation and hormone treatment groups in either Experiment, but follicular wave emergence was more synchronous in both treatment groups compared to the untreated control phase. The second study was conducted to develop an efficient method for ovarian superstimulation and oocyte collection during the anovulatory and ovulatory seasons. During the anovulatory season, one experiment was conducted in two replicates to compare the superstimulatory effect of 2500 IU of eCG (n = 10) given intramuscularly vs two doses of 200 mg of pFSH each (n = 10) given subcutaneously. Additionally, the effect of 25 mg of pLH given 24 hours prior oocyte collection on oocyte quality and collection rate was evaluated for each superstimulatory treatment. Results showed that treatment with pFSH induced a higher superstimulatory response and more cumulus oocyte complexes (COC) collected than did eCG during the anovulatory season. Furthermore, treatment with pLH increased the proportion of expanded COC that were collected with ultrasound-guided follicular aspiration. Two experiments were conducted during the ovulatory season, to develop an efficient protocol for superstimulation and oocyte collection. In Experiment 1, we compared the effect of two

intramuscular doses of 200 mg of pFSH in saline (n = 11) vs two intramuscular doses of 200 mg of pFSH in a proprietary slow release formulation (SRF; n = 11). In Experiment 2, we compared the effect of a single dose of 2500 IU eCG intramuscularly vs two doses of 200 mg of pFSH administered subcutaneously. Results showed that a 2-dose regime of pFSH, diluted in either saline or a slow-release formulation induced a similar superstimulatory ovarian response in wood bison, while bison given a single-dose of 2500 IU eCG had a significantly lower ovarian response. In summary, synchronization of follicle wave emergence can be effectively accomplished in wood bison during the anovulatory season and follicular ablation, E-17β and E-17β + P_4 treatments all shortened, and decreased the variability in the interval to follicular wave emergence. In addition, oocyte collection by transvaginal ultrasound-guided follicle aspiration from superstimulated bison was feasible and practical. Finally, treatment with pFSH was more effective than eCG to induce ovarian superstimulation for ultrasound-guided follicle aspiration in wood bison during both the anovulatory and ovulatory seasons.

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DEDICATION

I want to first dedicate this thesis to my parents, Demetrio and Luzmila, who brought me up with their love and wisdom to make me understand the importance of having a career and to be a professional.

To my brothers Wilder, Mary, Luis, and Armando, my nieces Stephanie, Jennifer, and Lady, my nephew Gustavo, my sister-in-law Marisol and my brother-in-law Gustavo for always trusting me and giving me their truly love and support.

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LIST OF ABBREVIATIONS

AI	Artificial insemination
cc	Cubic centimetre
CIDR	Controlled internal drug releasing device
CL	Corpus luteum
COC	Cumulus-oocyte complexes
COSEWIC	Committee on the Status of Endangered Wildlife in Canada
eCG	equine chorionic gonadotropin
EINP	Elk Island National Park
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
HLWBRP	Hook Lake wood bison project
IM	Intramuscular
IOI	Interovulatory interval
IWI	Interwave interval
LH	Luteinizing hormone
MHz	Megahertz
mg	Milligram
ml	Millilitre
mm	Millimetre
P4	Progesterone
PGF2α	Prostaglandin F2α
SEM	Standard error of the mean
SQ	Subcutaneous
vs	Versus
WBNP	Wood Buffalo National Park

1. GENERAL INTRODUCTION

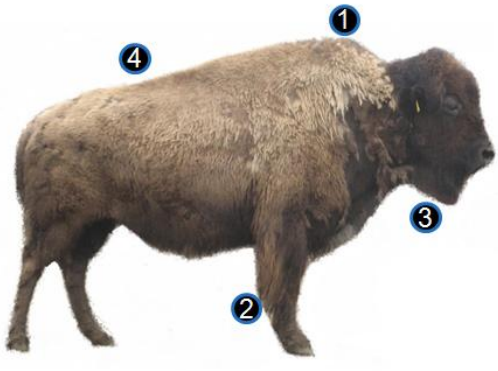
1.1 The North American bison.

The North American bison (*Bison bison*), along with the European bison (*Bison bonasus*), are species that belong to the family Bovidae, a group of mammals which also includes cattle (*Bos taurus)* among others (Reynolds *et al*., 2003). Otherwise known as American buffalo, the North American bison is the largest land mammal in North America. Within the species there are two subspecies: the wood bison (*Bison bison athabascae*) and the plains bison (*Bison bison bison*). Wood bison and plains bison resemble each other, but there are some genetic and phenotypic differences between them (van Zill de Jong *et al.,* 1995; Wilson and Strobeck, 1999). Among the external characteristics, the two subspecies can be differentiated by body size, location of the highest point of the hump, hair growth on the front legs (chaps), cape, and tail (Fig. 1.1).

Bison are gregarious animals and form complex groups based on a linear dominance hierarchy where the dominant females are usually the heaviest, fastest and with less hair (Vervaecke *et al*., 2005). Factors related to their aggregation and foraging behavior include season, sex, social status, and forage availability (Reynolds *et al*., 2003).

Studies have shown that bison herds originally came from Asia via the Bering Land Bridge. Bison evolved, changed their body conformation, and became distributed throughout North America, from Alaska to northern Mexico (McDonald, 1981). Currently, bison populations in dispersed groups are located in national reserves, private ranches, and zoos within Canada, USA, and Mexico (Reynolds *et al*., 2003).

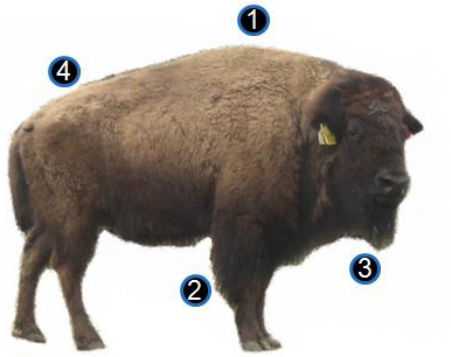
1.2 Importance of bison



Female wood bison

(Bison bison athabascae)

- Larger and heavier
1. Highest point is forward of the forelegs
2. Reduced or absent chaps
3. Smaller thin beard
4. More abrupt change of contour



Female plains bison

(Bison bison bison)

-Smaller and stockier
1. Highest point is on or behind the foreleg
2. Well developed chaps
3. Larger more rounded beard
4. Declining

Figure 1.1 A comparison of some phenotypic characteristics of wood bison and plains bison. Based on information from van Zyll de Jong *et al*. (1995).

Bison have been an important part of the Native American community and their culture and economy, because multiple products obtained from bison (i.e., meat, hide, and wool) have been used by these people to survive (Berger and Cunningham, 1994; Haines, 1995; Garret, 2007). After being nearly exterminated by settlers and hunters, American bison are in process of recovery; however, the majority of bison herds are now privately owned. Despite this, bison and First Nations people still share a close relationship which also includes their surrounding lands and environment (Garrett, 2007).

Of the estimated population of 500,000 bison in North America, 95% are on private ranches and farms. The most common commercial products obtained from bison are meat, viscera, rawhide, wool, and bones (National Bison Association, 2011). In addition, some producers sell sires, cows, and calves to other producers wanting to establish themselves in the bison industry

(Dineen, 2010). To earn extra income some private ranches offer a variety of agritourism activities that allow visitors to learn about the importance of bison conservation and their importance in preservation of the environment (Matheson, 2010).

An estimated population of 20,000 bison are grazing freely in public and protected areas of Canada and the United States (National Bison Association, 2011). These ruminants contribute to the preservation of the fragile balance of the ecosystem. The ecological functions of North American bison include being a prey for predators (e.g. wolves), providing an important nutrient source for scavengers, interacting and competing with other ungulates (i.e. elk, deer, moose), creating landscapes through grazing and wallowing and providing wool for small mammals and nesting birds, etc. (reviewed in Sanderson *et al*., 2007). Interestingly, the interaction between bison grazing activities and fire has been reported as a keystone in the conservation and restoration of the tall prairie grass (Knapp *et al*., 1999).

Restoration of bison populations is complicated by two main problems: human management and endemic diseases. Anthropogenic selections for meat production and ease of handling and hybridization with cattle have been reported as current problems facing conservation of bison (Freese *et al*., 2007). Secondly, diseases introduced by cattle, such as tuberculosis and brucellosis, have contributed to the decrease in wild bison population due to a high mortality rate related to increased susceptibility to predation, and abortions (McCorquodale and DiGiacomo, 1985; Joly and Messier, 2001; Reynolds *et al*., 2003; Himsworth *et al*., 2010b). Dealing with these diseases has been an extremely complicated issue in cattle and is even more complicated in threatened species of animals such as the wood bison (Tessaro *et al*., 1990).

1.3 Wood bison and Wood Buffalo National Park (WBNP)

The historical range of wood bison included the majority of the northwest areas in North America such as Alaska, Yukon, northeastern British Columbia, northern Alberta, and northwestern Saskatchewan (Soper, 1941; van Zyll de Jong, 1986). The main habitat of wood bison is constituted by coniferous forests, grasslands, meadows and prairies which are abundant in the above mentioned areas (Larter, 1988; Reynolds *et al*., 2003). The population of wood bison at the beginning of the nineteenth century was estimated to be 168,000 (Soper, 1941). This number decreased dramatically until the late nineteen century when only 250 individuals were left in the wild in Canada. Overhunting, as occurred with plains bison in North America, was considered the main factor for their near extinction (Soper, 1941). The Canadian government made efforts to preserve wood bison and gave authority and responsibility to the Northwest Mounted Police (1894 – 1911) for the protection of remaining wood bison. Hunting was banned and poaching was controlled, which enabled an increase to 500 bison during that period.

Wood Buffalo National Park (WBNP) was established in 1922 to ensure a steady recuperation of the Canadian wood bison population (Environmental Assessment Panel, 1990; Gates *et al*., 2001). Located in the extreme north of Alberta and overlapping into the Northwest Territories, WBNP is the largest national park in Canada (44,807 km^2 or 17,300 mi^2). Between 1925 and 1928, a total of 6673 plains bison were moved to the WBNP from the Wainwright Buffalo Park to address the problem of overpopulation of plains bison in that reserve (Environmental Assessment Panel, 1990). Unfortunately, this resulted in the introduction of cattle diseases (i.e. tuberculosis and brucellosis) and hybridization with native wood bison (Soper, 1941; Tessaro *et al*., 1990). In the early 1970's, the bison population in the WBNP was estimated to be 16,000 individuals. Since then, numbers have been declining, and in the late 1990's only 2,300 bison were found in the reserve (Joly and Messier, 2001; Mitchell and Gates,

2002). The combined effects of cattle diseases (brucellosis and tuberculosis) and predation by wolves (Joly and Messier, 2001) were considered the most important factors in the decline of the bison population. Therefore, the recovery and conservation of wood bison in the WBNP is of great importance, not only because this reserve contains the largest herd of wood bison in North America, but also because it is the most genetically diverse population of wood bison in Canada (McFarlane *et al*., 2006).

1.4 Endemic diseases: brucellosis and tuberculosis

1.4.1 Characteristics, diagnosis and importance in cattle

Bovine tuberculosis and brucellosis are two major diseases associated with large economic losses due to their chronic nature in cattle (Bernues *et al*., 1997). Bovine tuberculosis is a chronic infectious disease caused by *Mycobacterium bovis* (Garry, 2008). Important clinical signs of turberculosis include progressive emaciation, chronic moist cough, and palpable lymph node enlargement (Garry, 2008; Radostits *et al*., 2007). Losses in productivity in cattle stem from reduced milk and meat production (Zinsstag *et al*., 2006). Indirect losses include costs of testing, fencing, record keeping, and herd losses (Buhr *et al*., 2009). Bovine brucellosis is a bacterial disease caused by *Brucella abortus* and is characterized by late-stage abortions, death of newborn calves, retained placenta with the subsequent uterine infections and secondary infertility, as well as synovitis, orchitis and epididymitis (Garry, 2008; Olsen and Tatum, 2010). Abortions and decreased milk production as well as an increase in the intercalving interval are the main losses in cattle (Radostits *et al*., 2007).

Bovine tuberculosis and brucellosis are important zoonoses that threaten public health due to the interrelation among livestock animals, their products and humans. Therefore, efficient diagnosis and elimination of infected animals are important actions in any eradication program

(Beran, 1994). The traditional test for diagnosis of tuberculosis in cattle is the caudal-fold tuberculin test (Garry, 2008). However, fluorescent polarization assay, multiantigen print immunoassay, and DNA amplification–based rapid test were used recently in bison as complementary assays (Himsworth *et al*., 2010b). Agglutination tests, complement fixation tests, and enzyme linked immune assays have been used for diagnosis of bovine brucellosis, but all have problems with sensitivity and specificity (Nielsen, 2002). More recent methods to diagnose brucellosis include outer membrane protein typing, amplified fragment length polymorphism, and chromosomal indexing of DNA polymorphisms to determine the strain involved in the disease (Cutler *et al*., 2005).

In 1985, Canada was declared brucellosis free in domestic cattle (Environmental assessment panel, 1990) and tuberculosis has also been eradicated from cattle herds in all regions of the country since 2005 (Koller-Jones *et al*., 2006). However, sporadic and isolated cases of these diseases have occurred in some regions of Canada, suggesting that brucellosis and tuberculosis remain in some wildlife reservoirs (Nishi *et al*., 2006; Olsen, 2010). Re-emergence of endemic diseases that were eradicated in cattle is likely due to the complex interrelationship between wildlife and domestic animals; therefore, wildlife reservoirs of these diseases threaten disease-free populations and force researchers to address the problem in an interdisciplinary way (Rhyan and Spraker, 2010).

1.4.2 Wildlife reservoirs

Tuberculosis and brucellosis have been found in wildlife throughout the world. Badgers are known to be infected with *Mycobacterium bovis* in Switzerland, England, and Ireland. Tuberculosis is also found in ferrets in New Zealand and Australia (Clifton-Hadley *et al*., 2001) and brucellosis occurs in wolves, coyotes, feral swine, seals, dolphin, lagomorphs and rodents

around the world (Davis, 1990; Thorne, 2001). However, bison, deer species, pronghorn antelope, African buffalo, and other wild ungulates are considered the most important wildlife reservoir in the maintenance of both tuberculosis and brucellosis (Cheville *et al*., 1998; Clifton-Hadley *et al*., 2001; Davis, 1990; Nishi *et al*., 2006; Thorne, 2001). In North America, bison (*Bison bison*) and wapiti (*Cervus elaphus*) are reported to be the two primary reservoirs of tuberculosis and brucellosis (Essey and Koller, 1994; Nishi *et al*., 2006; Olsen, 2010). The risk of reintroduction of tuberculosis and brucellosis into domestic herds is particularly high due to the persistence of these diseases in wildlife reservoirs.

1.4.3 Brucellosis and tuberculosis in Canadian wood bison

Wood Buffalo National Park (WBNP) in northern Alberta and Riding Mountain National Park in south-western Manitoba are the main wildlife reservoirs for *Mycobacterium bovis* and *Brucella abortus* in wildlife populations. Both diseases are maintained in wood bison as the primary reservoir in the WBNP (Tessaro, 1986; Lees *et al*., 2003).

Bovine tuberculosis was first recognized in bison in WBNP in 1937, and then confirmed between 1952 and 1956 after inspection of slaughterhouse carcasses (Tessaro, 1986). Since then, tuberculosis has been considered endemic in free-roaming bison herds in and around WBNP and currently has an estimated prevalence of 49% (Joly and Messier, 2004a). Bovine brucellosis was first reported in WBNP in 1955-1956 after slaughter and inspection of three bison (Corner and Connell, 1958). Although suspected, it was not possible to confirm that the disease was brought into WBNP from plains bison translocations of the 1920's (Environmental Assessment Panel, 1990). The estimated prevalence of brucellosis in bison in WBNP is 31% (Joly and Messier, 2004a).

Semi-captive bison in reserves located around WBNP, such as Mackenzie Bison Sanctuary and Elk Island National Park, are considered free of tuberculosis and brucellosis; hence, infected bison in WBNP represent a threat to these populations (Gates *et al*., 2001). Without intervention, tuberculosis and brucellosis will likely persist indefinitely in WBNP. Until appropriate measures are taken to control these diseases, the proximity between infected populations and other animals represents an inevitable cause of future outbreaks (Joly and Messier, 2004a, Nishi *et al*., 2006; Tessaro *et al*., 1990).

1.4.4 Impact of endemic disease on the wood bison population

The effects of brucellosis and tuberculosis in bison are considered similar to those described in cattle. Bison seropositive to *brucella abortus* experienced abortion, retained placenta, orchitis, epididymitis, bursitis, arthritis, and reduced joint mobility (Tessaro, 1986; Williams *et al*., 1997). Likewise, necropsy revealed lesions typical of tuberculosis in emaciated and weak bison that were also positive to the tuberculin test (Tessaro *et al.*, 1990; McCormack, 1992; Joly and Messier, 2001)

Brucellosis and tuberculosis could affect the dynamics of wood bison populations in several ways. Their impact on reproduction has been studied extensively (Joly, 2001). It was reported that 50% of bison exposed to bovine brucellosis aborted and that mortality of newborn ocurred due to infection with *Brucella abortus* (Davis, 1990). In addition, a low probability of pregnancy was found in tuberculosis-positive bison (Joly and Messier, 2005). It has been suggested that bison infected with tuberculosis and brucellosis are more likely to abort during the winter and have less chance of surviving the winter than healthy bison (Joly, 2001; Joly and Messier, 2005).

Brucellosis and tuberculosis can also increase the risk that bison are caught by their predators. Bison represent a primary prey species for wolves (Reynolds *et al*., 2003) in WBNP, and it is thought that the decline of bison population is related to the index of wolf population (Carbyn *et al*., 1998). Although a significant relationship between bison and wolf populations was reported, it is well known that wolves have other sources of food (e.g. moose) in the park. Therefore, it is unlikely that wolf predation alone would have provoked a dramatic population decline of wood bison in WBNP (Joly and Messier, 2000). Other studies have suggested a possible interaction between endemic disease and predation in the decline of bison population. Joly and Messier (2004b) found that bison herds infected with bovine tuberculosis and brucellosis have a high probability (68.5%) of being hunted by wolves. Indeed, weight loss from reduced foraging efficiency, and lesions provoked by these diseases (e.g. swollen stifle joints and arthritis) increase the risk of predation (Joly and Messier, 2001). Conversely, disease-free bison herds have low incidence of predation by wolves (Joly and Messier, 2004b). These findings support the hypothesis that the interaction between endemic disease and predation since the 1960s is responsible for the decline in the wood bison population in WBNP (Joly and Messier, 2001; Joly and Messier, 2004b).

Other contributing factors to the population dynamics of wood bison include accidents and climate change. Drowning and extreme snowfalls have been associated with mortality in bison (Soper, 1941). In 1974, thousands of bison drowned during a very rare spring flood which occurred in the delta region of WBNP (Reynolds *et al*., 2003). Likewise, hard winters with unusually deep snow will reduce the availability of forage for bison, thus affecting their body condition and susceptibility to disease and predation (Environmental Assessment Panel, 1990; Reynolds *et al*., 2003).

1.5 Wood and Plains Bison hybridization

The translocation of plains bison to WBNP 80 years ago was a controversial decision and posed the risk of hybridization between wood bison and plains bison (Soper, 1941). Consequently, several years later, a number of bison herds in the WBNP were composed of hybrids of wood and plains bison (van Zyll de Jong, 1986, van Camp, 1989). In 1959, a small group of pure wood bison located in the Nyarling River, north of WBNP, were captured and tested for tuberculosis and brucellosis. Based on negative test results, these disease-free pure wood bison were transferred to other disease-free areas to ensure the survival of the herd (van Camp, 1989). Although it was reported that wood bison from the WBNP remain genetically and morphologically different from plains bison (van Zyll de Jong *et al*. 1995), further studies have suggested that all wood bison in and around the WBNP contain some plains bison genetic material (Wilson and Strobeck, 1999). Efforts to maintain pure wood bison herds are threatened by an incursion of plains bison ranching around the park. Escape of captive plains bison and hybridization with free-roaming wood bison are major concerns (Harper *et al.*, 2000).

1.6 Alternatives for the recovery of wood bison in WBNP and Canada

In 1979, the Committee on the Status of Endangered Wildlife in Canada (COSEWIC) listed wood bison as “Endangered” (Environmental Assessment Panel, 1990) as a measure to protect the remaining population. Later, in 1988, COSEWIC listed wood bison as a “Threatened” species by recommendation of the Wood Bison Recovery Team (Mitchell and Gates, 2002), a status that was confirmed by COSEWIC in 2000.

The National Recovery Plan for the wood bison was prepared to identify recovery actions that would help to change the legal status of wood bison (Gates *et al*., 2001). The recovery plan has the specific objectives of maintaining the genetic integrity of wood bison populations,

achieving the best possible recovery of the wood bison, and encouraging management programs to prevent the occurrence of serious diseases in wood bison herds (Environmental Assessment Panel, 1990). A list of strategies, actions, and schedules for the reclamation of wood bison are compiled in the recovery plan (Gates *et al*., 2001).

Reserves and National Parks have been established to conserve disease-free bison herds in Canada. The translocation of 16 wood bison negative for both tuberculosis and brucellosis to the Mackenzie Bison Sanctuary in 1963 was also implemented to preserve disease-free wood bison in the Northwest Territories (Environmental Assessment Panel, 1990; Nishi *et al*., 2002b). The total population of bison within the Sanctuary increased over the years, reaching an estimated in 1600 individuals in 2008, considered the largest disease-free wild population of wood bison presently in Canada (Mitchell and Gates, 2002). Similarly, captured bison were transferred to Elk Island National Park (EINP) in central Alberta between 1965 and 1968; and after a rigorous management protocol involving isolation and quarantine of neonates, the EINP breeding herd was declared tuberculosis and brucellosis free in 1971 (Gates *et al*., 2001). The park has a captive group of 350 wood bison and is managed as a source population for the creation of other disease-free herds in Canada (Mitchell and Gates, 2002; Nishi *et al*., 2002b). Interestingly, 29 wood bison were traslocated from EINP to the Hay-Zama area, in north-western Alberta in 1981, and the population has steadily increased to around 750 individuals that are negative to brucellosis and tuberculosis tests (Gates *et al*., 2001; Mitchell and Gates, 2002). In 1996, the Hook Lake Wood Bison Project (HLWBRP) established a disease-free, captive, wood bison herd in the Slave River lowlands, Northwest Territories, Canada; status still maintained in 2002 (Nishi *et al*., 2002a). But in 2005, an outbreak of bovine tuberculosis was reported in a HLWBRP captive herd, and although the origin of the outbreak was not clear, there were four potential

routes by which *Mycobacterium bovis* could have been transmitted to the captive herd. These included transmission of this bacteria via fomites (i.e. contaminated clothing), contamination of the isolation facility prior to, or during its construction, introduction through wild animal vectors (i.e. white-tail deer), and undetected infection in one of the founder animals translocated to HLWBRP (Himsworth *et al*., 2010a).

The latent risk of spread of tuberculosis and brucellosis from infected populations in and around WBNP to disease-free wood bison herds in this (Tessaro *et al*., 1993; Gates *et al*., 2001) and other areas presents an issue that needs to be addressed appropriately in Canada (Mitchell and Gates, 2002). The Environmental Assessment Panel recommended the eradication of the existing bison population in an around WBNP in 1988 to protect domestic cattle, disease-free wood bison and people in the area, and to repopulate the area with healthy wood bison from disease-free areas or obtained through the use of salvage operations (e.g. reproductive technologies) (Environmental Assessment Panel, 1990). In this respect the effective application of assisted reproductive technologies in wood bison depends upon a basic and detailed understanding of the normal reproductive biology of this species.

1.7 Reproductive physiology in bison

The development of the use of assisted reproductive technologies in a particular species is dependent on a complete understanding of the estrous cycle, reproductive physiology, and hormonal mechanisms involved (Mapletoft *et al*., 2009; Bertolini and Bertolini, 2009). To date, the reproductive physiology in bison is not as well documented as in cattle, a species in which there has been a rapid utilization of assisted reproductive technologies to increase reproductive efficiency and genetic merit in the last several years. The use of assisted reproduction

technologies in cattle has provided an invaluable model for the application of these technologies in endangered species (Solti *et al*., 2000).

1.7.1 Reproductive tract in the female bison

The general morphology of bison reproductive organs and their disposition in the pelvic cavity are very similar to that of dairy or beef cattle (Haigh *et al*., 2000). Therefore, the same criteria for reproductive evaluation may be used. As in cattle, rectal palpation is effective for evaluation of female internal organs (Haigh *et al*., 1991). The reproductive system of bison cows is composed of the ovaries, oviducts, uterine horns, uterus body, cervix, vagina and vulva; the differences with respect to cattle can be attributed to the size of adult females (Haigh *et al*., 2000).

1.7.2 Sexual maturation

Puberty is usually defined as the point in time that an animal achieves the ability to release viable gametes and display complete sequences of sexual behavior. In neuroendocrine terms, puberty in the female may be viewed as activation of gonadotropin-releasing hormone (GnRH) secretion (Ebling, 2005) with subsequent follicle development, ovulation and normal luteal function.

Bison cows may reach puberty as early as 12 months of age, but the onset of puberty depends on several different factors including, season, and nutrition (Green and Rothstein, 1991). However, in males, age has been determined as the main factor for the onset of puberty (Helbig *et al*., 2007). Talking into account this study bison bulls reach puberty at 16 months and they have to be 24 months old to be considered a competent breeder. Similarly, the age at first

breeding in females varies depending on geographic location and nutritional condition, and determines age at first calving (Reynolds *et al*., 2003). However, few bison conceive as yearlings; e.g., parturition was reported to occur in only 5% of 2-year-old females at WBNP (Fuller, 1966), and 12% of 2-year-old females at Wichita Mountain Wildlife Refuge (WMWR), Oklahoma, USA, (Shaw and Carter, 1989). The calving rate for 3-year-old bison was 52% at WBNP (Fuller, 1966), and 73 to 87% at WMWR (Shaw and Carter, 1989). The relationship between growth and reproduction also depends on the age and although a negative relationship between post-pubertal growth and reproductive performance was observed, the long-term reproductive success was greater for early-maturing females despite their reduced growth (Green and Rothstein, 1991).

1.7.3 Neuroendocrine control of ovarian function

The neuroendocrine system is responsible for regulating the majority of reproductive functions in mammals, but very little has been published about it in bison. This system in females includes the hypothalamus, anterior pituitary, ovaries and uterus; and reproductive hormones secreted to control physiological reproductive changes (Yen, 1999). Gonadotropin releasing hormone (GnRH), gonadotropins, steroid hormones, and prostaglandin are involved in the neuroendocrine control of reproduction.

The hypothalamus produces GnRH, a decapeptide that plays a key role in the regulation of mammalian reproduction (Edqvist, 1993). In estrous-cycling females, GnRH is secreted and released in a pulsatile manner into the hypophysial-portal circulation and stimulates GnRH receptors in the anterior pituitary to synthesize and release gonadotropin hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH). GnRH and gonadotropins are released in an episodic manner. The secretory pattern has been characterized as either low amplitude and

high frequency pulses (phasic pattern) or by high amplitude and low frequency patterns (tonic pattern) depending of the moment in the cycle and its feed-back mechanisms (Levine *et al*., 1995; Fink, 2000). Both gonadotropins are glycoproteins that govern and control ovarian activity. FSH stimulates the growth of ovarian follicles while LH is responsible for follicle growth and the occurrence of ovulation and corpus luteum formation (see chapter 1.7.7). In addition, steroid hormones are synthesized in the ovary under the synergistic action of both FSH and LH (Yen, 1999; Cheng and Leung, 2000).

Estradiol-17β is the most important estrogen compound in cattle. It is secreted by the granulosa cells of follicles and controlled by the positive action of gonadotropins and negative action of inhibin (Yen, 1999; Fink, 2000). Progesterone, another important steroid hormone, is produced mainly by the corpus luteum under an initial stimulus of LH (Pineda, 2003; O'Malley and Strott, 1999). The physiological action of estradiol-17β is the maintenance of the normal morphological and functional status of female reproductive organs, whereas progesterone is responsible for the establishment and maintenance of pregnancy (Stevenson, 2007).

In relation to hormones secreted by the uterus, prostaglandin F2α affects the reproductive system in mammals, being responsible for the regression of the corpus luteum or luteolysis (Pineda, 2003). Luteolysis starts with the secretion of oxytocin from the corpus luteum which goes to the endometrial tissue in the uterus. There, oxytocin binds its receptors which stimulate the production and secretion of prostaglandin F2α in the endometrium. In ruminants, prostaglandin F2α arrives to the ovary via systemic circulation or a local veno-arterial mechanism (McCracken *et al*., 1999). If pregnancy occurs, the embryo trophoblast secretes recognition signals (e.g. interferon tau) which interrupt the production of prostaglandin F2α, thus avoiding its luteolytic effects (Bazer *et al*., 1998).

The hypothalamic-pituitary-ovarian axis controls ovarian activity by releasing GnRH and gonadotropic hormones (LH, FSH) that direct the functional status of the ovaries, whereas ovarian steroids exert positive or negative feedback on the hypothalamus and pituitary controlling the secretion of their hormones (Yen, 1999; Stevenson, 2007). Positive feedback occurs during the follicular growth where increasing levels of estradiol act on the axis causing increased release of pituitary LH and FSH (Speight *et al*., 1981). On the contrary, low estradiol levels and high levels of progesterone exert negative feedback on the axis throughout the corpus luteum lifespan. Therefore, high progesterone enhances the negative feedback of low levels of estradiol and low progesterone facilitates the positive feedback of estradiol in eliciting the LH surge and ovulation (Edqvist, 1993). Details of these processes are presented in chapter 1.7.7.

This pattern of reproductive control occurs in the majority of annual polyestrous mammals (Edqvist, 1993). However, in seasonal breeders such as horses and sheep, this mechanism of neuroendocrine control of reproduction is similar during the breeding season, but differs during seasonal anestrus (Thiery *et al*., 2002; Donadeu and Watson, 2007).

1.7.4 Reproductive Seasonality

Female bison are considered seasonally polyestrous (Reynolds *et al*., 2003; Goodrowe *et al*., 2007). The breading season starts in the early fall (August-September) and is characterized by follicular and luteal activity. The ovulatory season ends in early spring (March-April) and is followed by a period of seasonal anestrus which is interrupted just prior to the next breeding season (Rutley and Rajamahendran, 1995). Based on the above, it is likely that seasonality in bison depends of changes in the day length or photoperiod because reproductive variations clearly occur in opposite seasons. However, the mechanisms that control seasonal reproduction in bison are unknown.

Seasonal reproduction is a process whereby most mammals in temperate climate change the pattern of their reproductive activity to reduce the effects of annual changes in temperature and food availability (Karsch *et al.*, 1984, Gündoğan *et al*., 2003). The purpose of this process is to ensure that offspring are born at an appropriate time of year when temperature conditions are more favourable and food is abundant (Thiery *et al.*, 2002; Malpaux, 2000). Bison, as well as sheep and some wild ungulates (e.g. wapiti), are considered short-day breeders because they show "rut" or mating behaviour in autumn when daylight is short, allowing for the birthing period to occur in a favourable time of the year (Thiery *et al.*, 2002).

Among several factors, photoperiod is considered to be the main factor that controls reproductive seasonality (Malpaux, 2000). This is because photoperiod is constant between years unlike other climatic variables such as temperature and rainfall (Karsch *et al.*, 1984). As a result, animals use photoperiod as a synchronizer of endogenous biological rhythms, resulting in ovulatory and anovulatory periods in a year (Thiery *et al.*, 2002)**.** Domestic species show more distinct seasonality with increasing latitudes (i.e., > 35º north or south), where changes in photoperiod are more pronounced (Malpaux, 2000). A long day consists of a 16- to 18-hour period of light exposure while a short day is characterized by 8 hour of light exposure. Importantly, melatonin secretion from the pineal gland is affected differently by exposure to light or darkness (Dahl *et al.*, 2000). The stimulus of light is captured by the retina and the information is conveyed to the pineal gland, which promotes the synthesis and release of melatonin from serotonin during the hours of darkness (Malpaux *et al*., 2001). Melatonin is considered the endocrine messenger that animals use to determine the length of day, and its final action is modulation of the release of GnRH (Hazlerigg *et al*., 2001; Malpaux *et al*., 2001).

As with other hormones, melatonin has several sites of action, making it difficult to study. However, the identification of high affinity receptors in the brain of several mammals has helped to understand its mechanism (Hazlerigg *et al*., 2001; Malpaux *et al*., 2001). Bittman (1993) first reported the pars tuberalis as the main structure for binding melatonin in the brain. However, it was shown that the pars tuberalis as a target for melatonin induced secretion of prolactin without any effect on LH secretion (Malpaux *et al*., 2001). The premamillary hypothalamic area is the main target for melatonin to control the neuroendocrine reproductive cycle in sheep (Lincoln and Maeda, 1992; Malpaux *et al.*, 1998).

Multiple signal pathways are activated by melatonin ligand-receptors, such as Ca^{2++}, cAMP-responsive element binding protein, protein kinase A, RFamide-related peptides, and kisspeptin (Masana and Dubocovich, 2001; Smith *et al*., 2008). Other neurotransmitters, such as catecholamines, opioids, excitatory amino acids, and thyroid hormones also act directly on hypothlamic neurons inhibiting or stimulating GnRH secretion. The action of these neurotransmitters has been identified in other seasonal breeders such as horses, sheep, and hamsters (Nagy *et al*., 2000; Thiery *et al*., 2002; Yasuo *et al*., 2009). Kisspeptin and RFamide-related peptides, which have opposing effects, are thought to act together to regulate the activity of GnRH neurons in different seasons, leading to the annual change in reproductive function during transitions between breeding and non-breeding seasons (Revel *et al.,* 2008; Smith *et al*., 2008; Clarke *et al*., 2009).

Experiments during the anovulatory season in ewes have shown that photoperiod governs response of the hypothalamo-pituitary axis to the negative feedback action of estradiol (Karsch *et al.*, 1993). Estradiol is a potent inhibitor of gonadotropin secretion, exerting its action specifically on LH pulse frequency during long days in ewes (Thiery and Malpaux, 2003). Thus,

estrous cycles cease when days become longer because of increased sensitivy to the negative feedback effect of estradiol on the hypothalamus, resulting in decreased LH secretion (Legan and Winans, 1981).

The negative feedback of estradiol on GnRH secretion starts with the activation of estradiol receptors. The cellular pathways of estradiol receptor action are not clear, but it was found that there are two types of estradiol receptors in the brain - α and β; α is the most abundant type in all reproductive tissues in most mammals, and β is predominant in mice (Arreguin-Arevalo *et al.,* 2007; Glidewell-Kenney *et al.*, 2008). In the ewe, a recent report indicated that estradiol binding to α receptor stimulates secretion of RFamide-related peptide in the hypothalamus, which inhibits GnRH secretion (Kriegsfeld *et al.,* 2010). Arreguin-Arevalo *et al*. (2007) also reported that estradiol binds the two receptors in the pituitary, directly mediating the negative feedback of estradiol on secretion of LH. Finally, estradiol stimulates dopamine neurons in the hypothalamus which inhibits GnRH and LH pulsatility in seasonal breeders during the non breeding season (Anderson *et al.,* 2001). In recent studies, it has been shown that estradiol exerts its effect by stimulating glutamate release onto A15 dopamine cells, which is responsible for inhibiting the GnRH surge in the brain of ewes during the anovulatory season (Singh *et al*., 2009).

1.7.5 Ovarian function during the anovulatory season

It was found that ovarian luteal activity ends in non pregnant bison in late April (Rutley and Rajamahendran, 1995). However, the mechanisms that induce the onset of seasonal anestrus in bison are not well known. Changes during transition from ovulatory to anovulatory seasons have been studied in sheep (Rawlings *et al*., 1977). No changes in circulating serum concentrations of LH, estradiol or progesterone were detected at the end of the last cycle. In addition, an

insufficient rise of LH secretion at the end of the last cycle of the season resulted in a failure of the dominant follicle to ovulate (Rosa and Bryant, 2003). In regards to follicular dynamics during the transition from ovulatory to anovulatory season in ewes, the endogenous rhythm of FSH decreases and the orderly emergence of follicular waves (follicles growing from 3 to ≥5 mm) cannot be maintained by this low level of FSH but then it returns to its normal secretion (Bartlewski *et al*., 1999).

Thyroid hormones have been implicated as regulators of seasonality in mammals on the period of transition from breeding season to seasonal anestrous (Nakao *et al*., 2008). These hormones affect the seasonal plasticity of GnRH neurons and that morphological changes in the GnRH neurons and glial processes may inhibit the seasonal secretion of GnRH and LH in mammals (Bernal, 2002; Malpaux, 2006; Nakao *et al.*, 2008).

Available information in seasonal breeders (e.g. wapiti) indicates that follicular waves occur during the anovulatory season (McCorkell *et al*., 2004). By means of transrectal ultrasonography, it was found that the growth of ovarian antral follicles occurs in a wave-like pattern throughout seasonal anestrus in wapiti. In addition, in bison, ovarian follicular dynamics during the anovulatory season has been characterized as wave-like development of antral follicles with the presence of a dominant follicle (McCorkell *et al*., 2008). The interval between the emergence of successive dominant follicles was reported to be 6.8 ± 0.6 days (mean ± sem). Moreover, the initiation of follicular growth was associated with temporary surges of FSH levels in blood circulation (McCorkell *et al*., 2008).

1.7.6 Ovarian function during the ovulatory season

The period of transition from the anovulatory season to ovulatory season in bison occurs during late summer and early fall. Based on fecal metabolites, the onset of breeding (ovulatory) season occurs in late July or early August in plains bison living in the Belgium Ardennes at 50º North latitude (Vervaecke and Schwarzenberger, 2006). However, a recent study involving the use of ultrasonography of a group of wood bison at 52º North latitude revealed that the ovulatory season began in late August (McCorkell *et al*., unpublished data). The first ovulation was followed by the formation of a poorly functioning, short-lived corpus luteum, resulting a short interovulatory interval (8 days; McCorkell *et al*., unpublished data). Although the luteal phase of this period is very short and lasts around 4 days (Vervaecke and Schwarzenberger, 2006), and the length of the estrus cycle is less than 10 days (Rutley and Rajamahendran, 1995), circulating levels of progesterone are elevated sufficiently to sensitize the hypothalamus-pituitary axis. Reproductive physiology during the transition to ovulatory season in bison seems to be similar to that reported in wapiti (McCorkell *et al*., 2007), and in post-parturition cows (Murphy *et al*., 1990).

The ovulatory season in bison extends from August to April (Rutley and Rajamahendran, 1995); however, the "rut", which is the mating period or the time where males and females are most sexually active, is short and occurs mainly between August and September (Reynolds *et al*., 2003). The ovulatory season is characterized by physiological events such as ovulation and corpus luteum formation (Goodrowe *et al*., 2007). Based on hormonal measurements in urine (pregnanediol-glucoronide) and feces (progesterone), the estrous cycle in bison lasts 21 (Matsuda *et al*., 1996) to 23 (Kirkpatrick *et al*., 1991) days. Likewise, daily examination of ovaries by ultrasonography revealed a pattern of two follicular waves during the estrous cycle, where the dominant follicle of the second wave ovulated (McCorkell *et al*., unpublished data). In addition,

nonpregnant females have cyclic luteal activity from the beginning of fall to the end of April (Rutley and Rajamahendran, 1995). In spite of the present information in bison, the reproductive physiology and its mechanism of control are not well documented. Therefore, the bovine species has provided an important model for the understanding of ovarian function in bison at this time.

1.7.7 Ovarian function in cattle

Traditionally, the ovarian cycle in cattle has been characterized by the occurrence of two phases: the follicular and the luteal phases. During the follicular phase, a dominant follicle and high levels of estradiol are predominant, whereas during the luteal phase presence of the corpus luteum (CL) and high levels of progesterone and estradiol predominate (Sirois and Fortune, 1988; Niswender *et al*., 2000). A process of growth and regression of antral follicles occurs during the ovarian cycle and leads to the development of a preovulatory follicle (Savio *et al*., 1988; Adams, 1999).

Follicular dynamics in all mammals studied to date occurs in a wave-like fashion which is characterized by the presence of two or three consecutive follicular waves in each estrous cycle (Ginther *et al*., 1989). A wave of follicular growth involves an initial recruitment of a group of growing follicles, of which one is selected and continues growing, while the others undergo atresia (Adams *et al*., 2008). The emergence of the follicular wave in response to a transient increase in the FSH is identified by the appearance of a cohort of small antral follicles of around 3 mm of diameter in cattle. The FSH peak occurs when the future dominant follicle reaches a size of about 4 mm and then circulating FSH levels begin to decrease. This suggests that a surge of FSH necessarily preceded the emergence of a wave (Adams *et al*., 1992a, Ginther *et al*., 1996). It is not clear why only one follicle is selected to be dominant, but when the largest

follicle in Bos Taurus cattle reaches 8.5 mm of diameter, it becomes the dominant follicle, while the other follicles become subordinates and regress (Ginther *et al*., 1997; Ginther *et al*., 2001). This is referred to as selection or deviation of growth profiles between dominant and subordinate follicles and a decline in circulating FSH levels trigger the selection mechanism (Adams *et al*., 1993). Although it is well known that the ovarian function is regulated primarily by the hypothalamus-pituitary hormones, it is also evident that locally produced factors, such as steroid hormones and growth factors, play an important role in the selection of the dominant follicles (Fortune, 1994).

The dominant follicle is the follicle which reaches the largest size in each follicular wave and is primarily responsible for exerting a suppressive effect on the development of subordinate follicles of the wave and preventing the emergence of a new follicular wave (Adams *et al*., 1993). There are several factors that appear to maintain the dominance of the largest follicle (Fortune, 1994). Estradiol and inhibin are hormones secreted by growing follicles, especially the dominant follicle, and have negative feedback on FSH levels (Adams *et al*., 1993). Likewise, the estradiol produced by the largest follicle or cohort is released into blood at the beginning of the deviation and suppress FSH to a level below that necessary to maintain the development of subordinate follicles (Fortune, 1994; Ginther *et al*., 2000). The nadir of FSH lasts around 3 days under the action of estradiol but the dominant follicle continues growing because its theca cells acquire more LH receptors and, its growth becomes more dependent on LH than FSH (Adams *et al*., 2008). The expression of LH receptors is greater in the largest follicle than in the second largest and it occurs before the follicle deviation (Beg *et al*., 2001). Likewise, if the suppressive effect of the dominant follicle on the small follicles is removed (e.g. follicular ablation), FSH levels rapidly increase resulting in the emergence of a new follicular wave (Ginther *et al*., 1999).

At the end of the growing phase, depending whether the corpus luteum regresses or not, ovulation may occur, stopping the secretion of estradiol, increasing FSH levels and triggering the emergence of the next wave, and therefore the ovarian cycle is repeated (Edqvist, 1993; Fink, 2000).

The length of the estrous cycle in cattle is related to the number of follicular waves that are present during an interovulatory interval. The estrous cycle last on average 21 days, but two follicular wave estrous cycles are somewhat shorter than three wave cycles (Sirois and Fortune, 1988). Emergence of the first follicular wave occurs on the day of ovulation (Day 0) for both 2- and 3-waves cycles. However, the emergence of the second follicular wave occurs on Day 9 or 10 for 2-wave cycles and on Days 7 or 8 for 3-wave cycles; the third follicular wave emerges on Days 15 or 16 (Adams, 1999; Adams *et al*., 2008; Jaiswal *et al*., 2009). During the luteal phase, follicular waves are under the influence of progesterone which determines that dominant follicles of successive waves undergo atresia. Interestingly, the suppressive effect of progesterone during the follicular growing phase has been shown to be in a dose-dependent manner when exogenous hormone is given (Adams *et al*., 1992b). In a 3-wave pattern, the size of the dominant follicle of the first wave is larger than in a 2-wave pattern, an event that is explained by the lower concentration of progesterone during the growth of the first wave dominant follicle in a 3-wave pattern (Adams *et al*., 1992b). Thus, the occurrence of 2- or 3-wave patterns would appear to be regulated by factors that influence the development of the dominant follicle of the first follicular wave. In addition, the lifespan of the CL influences the length of the estrous cycle; in the 2-wave pattern, the CL begins to regress on Day 16, but in the 3-wave pattern, the CL begins to regress on Day 19, resulting in a 19 to 20 day estrous cycle versus 22 to 23 days, respectively (Adams, 1999; Adams *et al*., 2008; Jaiswal *et al*., 2009).

1.8 Assisted reproductive techniques to develop a genetic resource bank in wildlife

Genetic resource banking is defined as the storage of gametes and embryos from valuable animals to be used in future programs of repopulation (Wildt, 1992). Genetic resource banks allow storage of semen, ova and embryos, as well as other tissues. The advantage of these banks is the preservation of the genetic variability of a species indefinitely (Holt *et al*, 1996, Wildt, 1992). The use of reproductive technologies has provided alternative solutions to facilitate the genetic management of some endangered populations (Solti *et al*., 2000). Synchronization, ovarian superstimulation, superovulation, *in vitro* embryo production, and cryopreservation are some of the technologies that can be used for the development of a genetic resource bank. However, the majority of studies about reproductive technologies have been done in cattle.

1.8.1 Ovarian synchronization

1.8.1.1 Induction of a new follicular wave in cattle

Follicular wave synchronization is necessary for any program of superstimulation or superovulation in cattle to ensure that the onset of superstimulatory treatments occurs at the time of the emergence of a new follicular wave (Nasser *et al*., 1993; Bo *et al*., 2002). Several methods to synchronize the follicular wave in cattle have been reported.

Gonadotropin-releasing hormone (GnRH) has been used to control follicular dynamics in cattle (Bo *et al*., 1995b; Pursley *et al*., 1995). GnRH induces the secretion of LH which will elicit the ovulation. With this, the suppressive effect of the dominant follicle on subordinate follicles is gone and a new wave emergence occurs (Pursley *et al.,* 1995). A single administration of GnRH significantly shortened the time (1.5 to 2 days) from GnRH treatment to emergence of a new follicular wave but was not enough to effectively synchronize the wave emergence (Bodensteiner *et al*., 1996; Martinez *et al*., 2000), apparently due to the stage of the dominant

follicle at the time of treatment. The dominant follicle is more likely to ovulate if GnRH is administrated at the late growing or early static phase than in other stages of follicle development (Martinez *et al*., 2000). Treatment with GnRH agonists (e.g. buserelin) has also been used for synchronizing wave emergence. Ovulation and a new wave emergence occur within 3 to 4 days after the administration of buserelin in cattle (Twagiramungu *et al*., 1995).

Steroid hormones have been used to induce a new follicular wave in cattle (Bo *et al.*, 1995b). For instance, estradiol has been used to synchronize follicular wave emergence in domestic animals (Adams, 1994; Bo *et al*., 1995b). In an early study, the administration of 5 mg estradiol valerate given one day after ovulation (early growing phase) in progestin-implanted cattle induced follicular atresia, resulting in an FSH surge and early emergence of the next follicular wave in heifers (Bo *et al*., 1993). Likewise, a single injection of 5 mg of estradiol-17β in cattle was effective in inducing follicular suppression and synchronous emergence of a new follicular wave 4 to 5 d later (Bo *et al*., 1994b). The action of the estradiol in synchronizing follicular wave emergence is not well understood, but it was reported that estradiol suppresses levels of FSH preventing the growth of FSH-dependent follicles (Adams, 1999). On the other hand, treatment with estradiol-17β + progesterone resulted in synchronous emergence of the next follicular wave 4 days later (Bo *et al.*, 1994b; Bo *et al.*, 1995a). The suppressive effect of the progesterone on LH pulses would appear to suppress the growth of LH-dependent follicles enhancing the action of estradiol (Savio *et al.*, 1993; Adams *et al*., 1992a; Bo *et al.*, 1995b).

Transvaginal ultrasound-guided aspiration of all follicles >4 mm has been shown to result in emergence of a new follicular wave 1.5 days after treatment in cattle (Bergfelt *et al.*, 1994; Martinez *et al.*, 2000). The aim of this technique is to remove the suppressive effect of all follicles, but especially large follicles on FSH release; therefore, following follicular ablation,

FSH surges and a new follicular wave emerges within 1 day (Bergfelt *et al.* 1994; Bo *et al.* 1995b).

1.8.1.2 Induction of a new follicular wave in bison

First attempts to synchronize the estrous cycles in bison were done by using Syncro-Mate-B (SMB implant) and estradiol valerate during the ovulatory season; however, only 45% to 50% of females displayed estrus 3 days after treatment ended (Matsuda *et al*., 1996, Othen *et al*., 1999). Later studies showed that the administration of 5 mg of estradiol-17β during the anovulatory season induced a new follicular wave 3 days after treatment, but unintended ovulation occurred after estradiol administration in 43% of treated females (McCorkell *et al*., 2010). Similarly, the use of an intra-vaginal progesterone-releasing device (Cue-Mate, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) and the administration of a single dose of estradiol 17β+progesterone resulted in the emergence of the new wave at 4.1 days after onset of treatment, and no ovulation after synchronization was reported (Adams *et al*., 2010). Likewise, ultrasound-guided follicular ablation resulted in a synchronous follicular wave emergence at 1.5 after ablation (McCorkell *et al*., 2010).

1.8.2 Ovarian superstimulation

1.8.2.1 Superstimulatory treatments in cattle

Ovarian superstimulation is the development of several ovarian follicles following the administration of follicle stimulating drugs. This method allows the rescue of subordinate follicles that otherwise would be lost due to atresia (Adams *et al*., 1993; Gordon, 2004). The objective of superstimulatory treatments in the cow is to obtain a maximum number of oocytes and transferable embryos with a high probability of producing pregnancies (Nasser *et al*., 1993;

Mapletoft *et al*., 2002). The extreme variability of the superstimulatory response due to intrinsic and extrinsic factors limits the usefulness of superstimulatory protocols. Intrinsic factors include age, breed, nutritional status, reproductive history, and ovarian status at the time of the treatment; while extrinsic factors include gonadotropin preparation used, total dose, duration and timing of treatment, FSH/LH activity of the drug, repeated treatments, season, and environment (Kafi and McGowan, 1997; Mapletoft *et al*. 2002). These factors have to be taken into account to establish an efficient protocol for superstimulation in cattle.

The most commonly used drugs to stimulate multiple follicular development are equine chorionic gonadotrophin (eCG) and follicle-stimulating hormone (FSH) (Goulding *et al.,* 1991; Mapletoft *et al*., 2002). Equine chorionic gonadotropin is a glycoprotein secreted by the endometrial cups of equine placenta. It has both FSH and LH effects and has a long circulating half-life of 3 to 5 days in cattle (Murphy *et al*., 1991). The structure and long lifespan of this hormone has been related to the induction of persistent follicles and abnormal endocrine profiles after exogenous eCG treatment (Mapletoft *et al*., 2002; Goulding *et al*., 1996). The use of eCG was reported to induce poor superstimulatory response in cattle (Monniaux *et al*., 1983; Sendag *et al*., 2008), response that could be related with the long half-life of eCG. Follicle-stimulating hormone, on the other hand, is a glycoprotein secreted by the anterior pituitary, and has been also used to induce ovarian superstimulation in cattle (Roover *et al*., 2005). The half-life of the FSH in the body is around 5 hours; thus, the traditional protocol of FSH requires multiple doses given twice a day for 4 to 5 days (Monniaux *et al*., 1983). Over the years, single and double subcutaneous injections of FSH have been used to induce ovarian superstimulation in cattle and results were considered equivalent to those obtained in the traditional twice-daily protocol of FSH (Bo *et al*., 1994a; Lovie *et al*., 1994; Alvarez *et al*., 2010). However, response to a single

subcutaneous injection of FSH in saline is dependent on sufficient subcutaneous adipose tissue to slow the absorption of FSH (Bo *et al*., 2010a). Therefore, single intramuscular injection protocols involving the use of a proprietary slow-release formulation have been developed (Bo *et al*., 2010a). Considering the stress associated with twice daily injections, less-frequent superstimulatory protocols may be considered more useful in wild species (i.e. bison) that are difficult to handle on a frequent basis.

Previous studies in cattle have revealed that ovarian superstimulation should be initiated at the time of follicular wave emergence to avoid the suppressive effect of the dominant follicle on subordinate follicles (Adams, 1994; Adams *et al*., 1994; Nasser *et al*., 1993). Small follicles of the new wave require FSH to continue their growth, to avoid selection of dominant follicle and to avoid the subsequent follicular atresia of subordinate follicles (Adams *et al*., 1993; Mapletoft *et al*., 1990). An optimal superstimulatory response occurs when treatment is initiated within 1 day of wave emergence (Nasser *et al*., 1993). Synchronization of follicle wave emergence by estradiol treatment or ultrasound-guided follicle ablation has been reported to be effective for superstimulatory purposes in cattle (Bo *et al*., 1994b; Bergfelt *et al*., 1997; Baracaldo *et al*., 2000).

1.8.2.2 Superstimulatory treatments in bison

The first attempt to apply a bovine protocol for superstimulation and superovulation was reported by Dorn *et al.* (1990). FSH was used as superstimulatory treatment and 5 morphologically normal embryos were collected from 10 female bison. The embryos were immediately transfered to synchronized cattle recipients, but no pregnancies were reported. In a later study, a single injection of 1500 IU of eCG was used in 3 bison cows, with an average of 4 ovulations per animal (Dorn, 1995). In another study, the effects of a single injection of eCG

(2500 IU) or FSH (400 mg mg NIH-FSH-P1) were compared; only a single female produced more than 1 CL (Othen *et al*., 1999). Thus, further studies are necessary in order to develop effective superstimulatory protocols that can be used in bison.

1.8.3 In vitro embryo production

In vitro embryo production (IVP) is a procedure to obtain embryos which involves three steps: *In vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) (Gordon, 2003). The IVM time of oocytes varies between 22 and 24 hours and can take place in conventional CO2 incubators in media covered with oil either in Petri dishes or in four-well dishes covered with oil. For IVF, a Petri dish containing several droplets of TALP (Tyrode's albumin lactate pyruvate) medium supplemented with antibiotic, amino acids, etc., are used to fertilize mature oocytes using a dose of 0.1 to 2 million sperm/mL (standard dose: 50,000 to 100,000 sperm per oocyte). IVF starts 18 to 26 hours after onset of IVM and oocytes are incubated in 5% $CO_2$and 20% O_2. After IVF, the presumptive zygotes are transferred to an IVC system for further development, where the system is similar to that described in IVF (Galli *et al*., 2001; Sirard and Blondin, 1996).

In cattle these steps are well established, but the variability of the number and quality of the oocytes collected still limit the large-scale use of the IVP. Oocytes for IVP can be obtained from slaughtered animals or live cattle on single or repeated occasions utilizing ultrasound-guided follicle aspiration (Galli and Lazzari, 1996; Gordon, 2003). Large quantities of oocytes are usually obtained at low cost by collecting abattoir ovaries which are used to establish the techniques as well as for a large-scale production of embryos. However, the major disadvantage of the oocyte collection from slaughtered ovaries is the unknown relationship between the

ovaries transported to the laboratory and the donors slaughtered at the abattoir (Gordon, 2003). In addition, wild or endangered species are not normally slaughtered in large numbers and slaughterhouses for wild animals (e.g., bison) usually are not close to embryo production labs and ovaries sometimes have to be sent long distances which would affect viability of oocytes.

Conversely, transvaginal ultrasound-guided follicle aspiration in cattle permits collection of oocytes from live animals on a repeated basis (Pieterse *et al*., 1988). Since the first use of transvaginal ultrasound-guided follicle aspiration, several groups have increased the number of oocytes retrieved, and the frequency of collection (Kruip *et al*., 1994). Briefly, a convex-array transvaginal ultrasound probe of 5 to 7.5 MHz, equipped with a needle guide is inserted into the vagina. Then an ovary is located by transrectal palpation and placed adjacent to the face of the probe placed against the vaginal fornix. Follicular aspiration and oocyte collection is performed by the introduction of a needle through the vaginal wall; the needle is attached to a container (e.g. EmCom filter) via tubing and suction is applied by using a vacuum pump (flow rate 20-30 ml/min) (Brogliatti and Adams, 1996; Hashimoto *et al*., 1998). The ultimate goal of the procedure is to produce a large number of embryos and pregnancies per donor cow over a given interval of time. Therefore, the combination of transvaginal ultrasound-guided follicle aspiration and *in vitro* embryo production may have a higher potential benefit than superovulation alone for embryo production (Galli *et al*., 2001).

Thundathil *et al*. (2007) first reported the successful use of IVP in bison. In this study, cumulus-oocyte complexes (COC) were aspirated from slaughtered ovaries and then oocytes were subjected to IVM, IVF, and IVC. For IVF, frozen-thawed and chilled epididymal sperm were used. Results showed a high cleavage rate (64% and 85%) and low blastocyst production rate (7.5% and 10%) for frozen-thawed and chilled sperm, respectively. To our knowledge, IVP

using oocytes obtained through transvaginal ultrasound-guided follicular aspiration has not been reported in bison.

Another issue that must be taken into account is the possibility of disease transmission through *in vitro*-produced embryos in bison. According to the sanitary protocols developed by the International Embryo Transfer Society, there is a low risk of transmission of pathogens by embryo transfer when procedures are strictly followed (Wrathall *et al*., 2006). It was suggested that the zona pellucida prevents infection of the embryonic material from external pathogens and that exposure to trypsin and antibiotics in the embryo media can remove viruses or kill bacteria respectively (Wrathall, 1995). However, there is always concern about the transmission of these pathogens attached to the zona pellucida (e.g., viruses) to diseased- free recipients, especially for those that come from in vitro-produced embryos (Stringfellow and Givens, 2000). To date, there have been no reports of infectious disease in cattle transmitted via embryo transfer through embryos that were collected *in vivo* or produced *in vitro* (Stringfellow and Givens, 2000; Wrathall *et al*., 2006).

2. GENERAL HYPOTHESIS

In pursuit of the overall goal of conserving the genetic diversity of the threatened wood bison population through the use of assisted reproductive technologies, we hypothesized that ovarian follicular synchronization and superstimulation, and transvaginal oocyte collection are feasible and practical techniques in wood bison, and may be applied during both breeding and non-breeding seasons.

3. GENERAL OBJECTIVES

To test this hypothesis, our objectives were to:

- Establish an efficient method of ovarian synchronization and superstimulation in bison.
- Determine the influence of season on ovarian response to ovarian superstimulatory treatments.
- Determine the effects of gonadotropin treatments on oocyte collection rates and quality in bison.

4. SYNCHRONIZATION OF FOLLICULAR WAVE DEVELOPMENT IN WOOD BISON (*Bison bison athabascae*)

4.1 Abstract

As part of an effort to develop a genetic resource bank to preserve Canada's threatened wood bison (*Bison bison athabascae*), the objective of this study was to establish an effective protocol to control follicular wave emergence in bison. Two experiments were done during the non-breeding season to compare the effectiveness of ultrasound-guided follicular ablation with estradiol and progesterone treatments. Bison cows (n = 19) were scanned ultrasonically once daily for 14 days to monitor ovarian follicular dynamics and establish a mean interval from the beginning of scanning to the emergence of a new follicular wave, that would serve as a control for comparison in Experiments 1 and 2. In Experiment 1, bison were assigned randomly to a follicular ablation group (n = 9), or a group given 2 mg estradiol (E-) 17β in oil (n = 10). In Experiment 2, bison were assigned randomly to a follicular ablation group (n = 9), or a group given 2 mg E-17β + 100 mg progesterone (P4; n = 10). Day 0 was designated as the day of follicular ablation or treatment. Follicular ablation consisted of transvaginal ultrasound-guided aspiration of all follicles ≥ 5 mm in both ovaries. Hormone treatment was given intramuscularly in the neck. The interval and variation in the interval to emergence of a new follicular wave was compared between the control phase and the two treatment groups by ANOVA and presented as mean ± SEM. In Experiment 1, the interval to new wave emergence for control, follicular ablation, and E-17β groups was 4.9 ± 0.66, 1.1 ± 0.11, and 3.1 ± 0.35 days, respectively ($P < 0.05$). The degree of synchrony was 2.4 ± 0.36, 0.2 ± 0.09, and 0.8 ± 0.20 days, respectively ($P < 0.05$). In Experiment 2, the interval to new wave emergence for control, follicular ablation and E-17β + P_4 groups was 4.9 ± 0.66, 1.2 ± 0.15, and 3.3 ± 0.33 days respectively ($P < 0.05$), and

the degree of synchrony was 2.4 ± 0.36, 0.2 ± 0.08, and 0.8 ± 0.19 days respectively ($P < 0.05$). The degree of synchrony did not differ between ablation and hormone treatment groups in either Experiment 1 or 2, but follicular wave emergence was more synchronous in both treatment groups compared to the untreated control phase. In summary, follicular ablation, E-17β and E-17β + P_4 treatments all shortened, and decreased the variability in the interval to follicular wave emergence in bison. In addition, follicular ablation produced an earlier emergence of a new follicular wave in bison than hormone treatments.

4.2 Introduction

Reclamation of Canada's threatened wood bison (*Bison bison athabascae*) in the Wood Buffalo National Park, Canada, is complicated by endemic diseases and hybridization with plains bison (*Bison bison bison*) (Mitchell and Gates, 2002; Nishi *et al.*, 2006). In 1990, the Environmental Assessment Panel concluded that eradication of the diseased wood bison herds would prevent the spread of endemic diseases, and suggested that pure wood bison herds should be isolated in different parks to help further repopulation through salvage techniques (Environmental Assessment Panel, 1990).

Reproductive technologies, such as cryopreservation of gametes and embryos provide the opportunity to develop genetic resource banks that can be used to facilitate genetic management of endangered wild animals (Wildt, 1992; Solti *et al*., 2000). However, effective use of these technologies depends on a basic understanding of the normal reproductive pattern of the species in question. The reproductive biology of bison has been partially characterized in studies of behavior, fecal and urine hormone measurements (Green and Rothstein, 1991; Dorn, 1995; Fuller *et al*., 2007; Vervaecke *et al.*, 2005). The length of the estrous cycle during the breeding

season was reported to be 21 - 23 days (Kirkpatrick *et al.*, 1991; Matsuda *et al*., 1996). However, a short cycle of 10 - 12 days was observed during the transition from the nonbreeding season to the breeding season (Vervaecke and Schwarzenberger, 2006). Recently, the use of serial ovarian ultrasonography during seasonal anestrus showed that follicular development occurs in a wave-like pattern, and the mean interwave interval was 7 days (McCorkell *et al*., 2008).

Knowledge of natural reproductive patterns in bison will be important for developing rational schemes for ovarian synchronization for the purposes of artificial insemination and superstimulation. Previous attempts to synchronize estrus in bison involved protocols designed for cattle based on the use of estradiol, prostaglandin, and progestin implants for 9 days (Dorn *et al.*, 1990; Matsuda *et al.*, 1996; Robison *et al.*, 1998; Othen *et al.*, 1999). Estrus in bison was observed at 3 to 4 days after the removal of the progestin implant. In cattle, treatment with a combination of estradiol-17β and progesterone resulted in synchronous emergence of the next follicular wave 4 days later (Bo *et al.*, 1994b; Bo *et al.*, 1995a). Alternatively, transvaginal ultrasound-guided aspiration of all follicles >5 mm resulted in emergence of a new follicular wave 1.5 days after treatment in cattle (Bergfelt *et al.*, 1994; Martinez *et al.*, 2000). Preliminary studies in bison revealed that treatment with estradiol-17β alone during the anovulatory season induced a new follicular wave, on average 3 days after treatment, but synchrony was confounded by unintended ovulation (McCorkell *et al*., 2010). In the same study, follicular ablation resulted in a synchronous follicular wave emergence, on average 1 day after ablation.

The objective of the present study was to develop a protocol to synchronize ovarian follicular wave emergence in bison during the anovulatory season. Specifically, we compared the efficacy of follicular ablation vs. treatment with estradiol-17β (Experiment 1), and follicular ablation vs. treatment with a combination of estradiol-17β + progesterone (Experiment 2).

4.3 Material and Methods

4.3.1 Animals

A total of 19 non-pregnant adult female bison were used during May and June (i.e., middle of the anovulatory season). Bison, with an average body condition score of 3 (scale of 1 – 5, from emaciated to fat; Vervaecke *et al*., 2005) were maintained on pasture at the University of Saskatchewan's Native Hoofstock Centre near Saskatoon, Saskatchewan (52°07′N, 106°38′W). They had free access to alfalfa/ryegrass hay and fresh water. The study was approved by the University of Saskatchewan's Animal Research Ethics Board, in accordance with the guidelines of the Canadian Council on Animal Care.

4.3.2 Control phase

To provide sufficient data for statistical interpretation, and to avoid later synchronizing effects of experimental treatments, data from a Control phase were used for comparison with data from Experiments 1 and 2. For daily examination, the bison were gathered in a large corral and separated into groups of 5 - 7 animals before bringing them into the bison handling facility. The bison facility consists of an outdoor alley leading into an indoor section divided into cells by several hydraulically controlled sliding gates which end in a squeeze chute (McCorkell *et al*., 2008). Bison were restrained individually in the chute in the standing position without the use of sedation. Beginning on May 18th, the ovaries of each bison were examined daily for 14 days by transrectal ultrasonography using a 7.5 MHz linear-array transducer (Aloka SSD 900, Tokyo, Japan). Feces were evacuated from the rectum and the probe was introduced into the rectum using a gloved hand. Both ovaries were scanned and follicles were recorded using the identity method (Knopf *et al*., 1989) which consisted of drawing a sketch of all follicles ≥ 4 mm in both

ovaries during each examination. Sequential ovarian follicular development was monitored until regression of a dominant follicle and emergence of a new follicular wave was evident. The day of the first examination was considered Day 0. The observational period encompassed at least one complete inter-wave interval (mean of 7 days; McCorkell *et al*., 2008). The mean and variation in the interval from the day examinations began to follicular wave emergence was calculated for each animal to serve as the non-treated control interval for comparison in other experiments.

4.3.3 Experiment 1

Immediately following the control phase, the 19 bison at random stages of the follicular wave development were assigned randomly to two groups and given 2 mg estradiol-17β in canola oil (1 mg/ml) intramuscularly in the neck (n=10) or underwent transvaginal ultrasound-guided follicular ablation (n = 9). Follicular ablation was done using a 5 MHz intravaginal probe (ALOKA SSD 900, Tokyo, Japan) equipped with a disposable 18 ga x 1 ½" vacutainer needle (BD Medical, Mississauga, ON, Canada) attached to a 10 ml syringe via silicone tubing (60 cm long x 1.14 mm internal diameter; Cole-Palmer, Montreal, Canada), as previously described (Bergfelt *et al.*, 1994). Females were restrained in the bison squeeze chute and a dose of 3 – 5 ml of 2% lidocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada) was given between the sacro-coccygeal space or first intercoccygeal junction to induce caudal epidural anesthesia. The vulva was washed three times with disinfectant and the probe was introduced into the vagina and placed in the fornix. Manipulation per rectum was done to position the ovary against the transducer face (i.e., separated only by the vaginal wall). All follicles ≥5 mm were aspirated through the vaginal wall and curettage of follicles was performed

by rotating the needle several times during aspiration. The disappearance of the follicle from the screen was considered effective follicular ablation (Bergfelt *et al*., 1994). Day 0 was considered the day of treatment (follicular ablation or estradiol-17β). Thereafter, the ovaries were examined daily as described in the control phase for a minimum of 7 days after treatment, or until emergence of a new follicular wave was evident.

4.3.4 Experiment 2

After an interval of ≥ 14 days from the end of Experiment 1, the same bison were assigned randomly to two groups and given either a combination of 2 mg estradiol-17β and 100 mg progesterone intramuscularly (prepared in canola oil at a final concentration of 1 mg/ml of estradiol-17β and 50 mg/ml of progesterone; n = 10), or underwent follicular ablation (n = 9) as described in Experiment 1). The ovaries were examined daily by ultrasonography to detect new wave emergence, as described in Experiment 1.

4.3.5 Statistical analyses

The interval from first examination (Control phase) or the day of treatment (Experiments 1 and 2) to new wave emergence was compared among groups (Control phase, hormone treatment, follicular ablation) by analysis of variance. A follicular wave was defined as the synchronous development of several antral follicles, culminating in the dominance of one of these follicles and regression of the subordinates (Ginther *et al*., 1989). Follicular wave emergence was defined as the day when the follicle destined to become dominant of the wave was first detected at a diameter of 4-5 mm (Ginther *et al*., 1989).The degree of synchrony or variability (the absolute difference between the group mean and individual values; Ratto *et al*., 2003) was compared

among groups by analysis of variance. Multiple comparisons between groups were made using Tukey post-hoc tests. Data analysis was carried out using the SAS version 9.2 (Cary, NC, USA).

4.4 Results

4.4.1 Control phase

The mean (± SEM) interval from the day of onset of scanning to the day of emergence of a new follicular wave was 4.9 ± 0.66 days, and the inter-wave interval was 7.7 ± 0.27 days. No ovulations or CL were detected during the Control phase.

4.4.2 Experiment 1

A female bison of the estradiol-17β group ovulated following treatment and was excluded from further analyses. The interval from treatment to a new follicular wave emergence did not differ between Control and estradiol 17β groups ($P = 0.11$), but the interval was shorter in the follicular ablation group ($P < 0.05$; Table 4.1). The variability in the interval to new follicular wave emergence was greater in the Control group ($P < 0.05$) compared with ablation and estradiol-17β groups. The degree of variability was similar in the ablation and estradiol-17β groups ($P = 0.51$). The diameter of the dominant follicle on Day 0 and at 7 days after emergence of the new follicular wave was similar among groups (Table 4.1). The effect of treatment on the new follicular wave emergence is shown in Fig. 4.1.

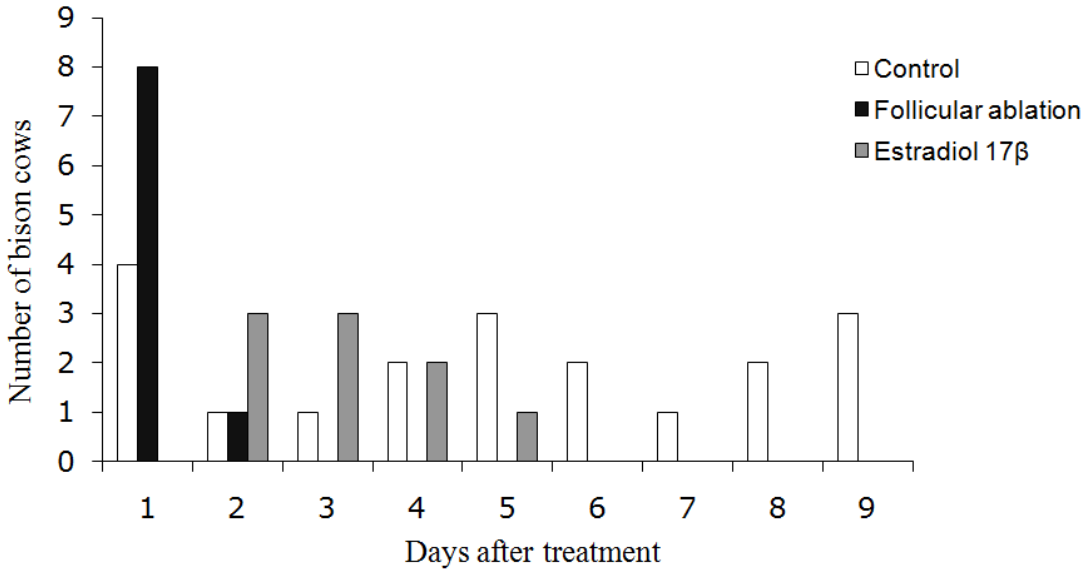


Fig. 4.1 Distribution of bison with new follicular wave emergence during the control phase and after transvaginal ultrasound-guided follicular ablation or estradiol-17β treatment.

Table 4.1. Effect of treatment on ovarian follicular wave synchrony in bison during the nonbreeding season (mean±SEM).

Groups	Interval to new wave emergence (days)	Variability in new wave emergence (days)	Dom. foll. at treatment (mm)	Largest follicle after wave emergence (mm)
Control (n = 19)	4.9 ± 0.66[a]	2.4 ± 0.36[a]	10.7 ± 0.28 [a]	11.4 ± 0.32 [a]
Follicular ablation (n = 9)	1.1 ± 0.11[b]	0.2 ± 0.09[b]	9.3 ± 1.15 [a]	12.0 ± 0.44 [a]
Estradiol-17β (n = 9)	3.1 ± 0.35[a]	0.8 ± 0.20[b]	9.7 ± 0.76 [a]	11.9 ± 0.56 [a]

[ab] Within columns, values with different superscripts are different ($P \leq 0.05$)

4.4.3 Experiment 2

The interval to the day of a new follicular wave emergence is shorter in the ablation group ($P < 0.05$) compared with control and estradiol-17β + progesterone groups ($P = 0.14$). The degree of synchrony in interval to follicle wave emergence did not differ between ablation and estradiol-17β + progesterone groups ($P = 0.65$), but both were more synchronous than the Control group ($P < 0.05$). As in Experiment 1, mean diameters of dominant follicle at the time of treatment and at 7 days after the day of new follicular wave emergence did not differ among groups ($P = 0.59$; $P = 0.45$, respectively). Figure 4.2 and Table 4.2 summarize results for all end points in Experiment 2.

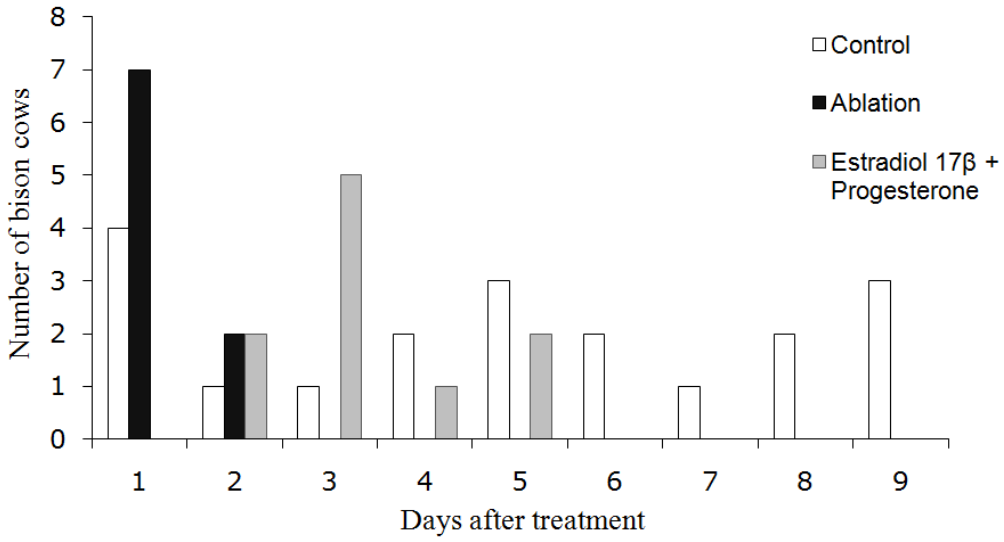


Fig. 4.2 Distribution of bison with new follicular wave emergence during the control phase and after transvaginal ultrasound-guided follicular ablation or estradiol-17β + progesterone treatment.

Table 4.2 Effect of treatment on ovarian follicular wave synchrony in bison during the nonbreeding season (mean±SEM).

Groups	Interval to new wave emergence (days)	Variability in new wave emergence (days)	Dom. foll. at treatment (mm)	Largest follicle after new wave emergence (mm)
Control (n = 19)	4.9 ± 0.66^{a}	2.4 ± 0.36^{a}	10.7 ± 0.28^{a}	11.4 ± 0.32^{a}
Follicular ablation (n = 9)	1.2 ± 0.15^{b}	0.2 ± 0.08^{b}	11.2 ± 0.94^{a}	11.7 ± 0.50^{a}
Estradiol-17β + P4 (n = 10)	3.3 ± 0.33^{a}	0.8 ± 0.19^{b}	11.3 ± 0.62^{a}	12.1 ± 0.53^{a}

[ab] Within columns, values with different superscripts are different ($P \leq 0.05$)

4.5 Discussion

Synchronization of follicular wave emergence would be an important tool for improving the utilization of assisted reproductive technologies such as artificial insemination or superstimulation in females (Solti *et al.*, 2000; Wildt, 1992). Various effective synchronization methods have been developed in domestic animals. The most effective approaches are physical or hormonal methods that seek to eliminate the inhibitory effect of the dominant follicle to initiate the emergence of a new follicular wave in a predictable interval of time (Adams, 1994; Bo *et al*., 1995b).

In the present studies, follicular ablation, estradiol-17β and estradiol 17β + progesterone treatments were investigated as synchronization protocols for bison. In addition, the research was performed in the middle anovulatory season (May - June) to ensure that females could be efficiently synchronized for gamete and embryo collection throughout the year.

The follicular ablation procedure consists in the ultrasound-guided aspiration of all follicles ≥ 5 mm in diameter, resulting in the emergence of a new follicular wave emergence in an

average of 1.5 days (Bergfelt *et al.*, 1994). The aim of this method is to remove the suppressive effect of large follicles on FSH release following ablation, circulating estradiol concentrations and its negative feedback effect on FSH decrease, allowing FSH to surge which elicits emergence of a new follicular wave within 1 day (Bergfelt *et al.*, 1994; Bo *et al.*, 1995b). A similar study, conducted in the non breeding season, was done in wapiti (McCorkell *et al*., 2008) where a new follicular wave emerged 1.4 days after performing follicular ablation. In preliminary studies in bison done in our lab follicular wave emergence occurred 1 day after follicular ablation (McCorkell *et al.*, 2010). Results of the present study are in agreement with those cited above; follicular ablation induced a new follicular wave emergence at 1.1 and 1.2 days in Experiments 1 and 2. These results suggest that it would be possible to use follicular ablation as a synchronization method for superstimulatory protocols in wood bison in the same way that is used in cattle as reported by Bergfelt *et al*. (1997).

Estradiol has been used as a steroid hormone treatment to synchronize a new follicular wave in domestic animals (Adams, 1994; Bo *et al*., 1995b; Meikle *et al.*, 2001). In cattle, 5 mg estradiol valerate given one day after ovulation (early growing phase) induced follicular atresia synchronizing the FSH surge and hastened the emergence of the next follicular wave (Bo *et al*., 1993). Likewise, a single injection of 5 mg of estradiol-17β in cattle was effective in inducing follicular suppression resulting in synchronous emergence of a new follicular wave 4 to 5 d later (Bo *et al*., 1994b). In addition, it is note-worthy that 5 mg of either estradiol valerate or benzoate resulted is longer and less synchronous wave emergence than 2 mg of estradiol-17B (Mapletoft *et al*., 1999; Thundathil *et al*., 1997; Colazo *et al*., 2002). In anestrous ewes, exogenous estradiol 17β caused the regression of the dominant follicle and subsequent emergence of a new follicular wave (Meikle *et al.*, 2001). Similarly, the administration of 5 mg estradiol-17β during the

anovulatory season in wapiti resulted in new follicular wave emergence 3.5 days after the injection (McCorkell *et al.*, 2008). The action of the estradiol in synchronizing follicular wave emergence is not well understood, but it was found that estradiol decreases levels of FSH preventing the growth of subordinate follicles (Adams, 1999). In any case, the administration of estradiol causes suppression of FSH levels which are followed in turn by an FSH surge and consequently follicle wave emergence (Adams *et al*., 1992a; Martinez *et al*., 2000; Colazo *et al*., 2003). So in essence, the synchronization of follicle wave emergence with estradiol involves the synchronization of the FSH surge.

Several studies have suggested that apoptosis can be the most important factor to induce follicular atresia; however, gonadotropins would play a critical role to prevent apoptosis in granulosa cells, where FSH is considered one of the main follicle survival factors (Yang and Rajahamendran, 2000a; Yang and Rajahamendran, 2000b). Therefore, we can speculate that changes in the secretion of FSH and/or LH due to estradiol treatments that were previously reported (Bo *et al*., 1993, Bo *et al*., 1994b) would be related to atresia of the largest follicles in domestic animals. In this regard, it was also reported that in ewes photoperiod governs the response of the hypothalamo-pituitary axis to the negative feedback action of estradiol (Karsch *et al.*, 1993). In fact, estradiol is a potent inhibitor of gonadotropin secretion, exerting its action specifically on LH pulse frequency during long days in ewes (Thiery and Malpaux, 2003). In our study, 2 mg of estradiol-17β induced the regression of the largest follicle and induced a new follicular wave at 3.1 days of the treatment. This result is in agreement with those previously reported in cattle and sheep, and recently in wapiti (Bo *et al.*, 1993; Barret *et al.*, 2008; Meikle *et al.*, 2001; McCorkell *et al.*, 2008) where a new follicular wave emergence was induced after estradiol treatment.

Results of a preliminary study in bison suggested that the synchronizing effect of estradiol-17β (5 mg) treatment was confounded by the induction of ovulation in 43% of treated females (McCorkell *et al.*, 2010). This result contrasts with our findings where ovulation occurred in only one animal. This can be explained because we used a lower dose of estradiol 17β (2 mg) compared with that reported by McCorkell *et al*. (5 mg, 2010). Apparently the higher dose of estradiol was sufficient to overcome the seasonal suppression of the hypothalamus and pituitary gonadotopes resulting in the secretion of GnRH and a surge release of LH. We suggest that the lower dose of estradiol (e.g., 2 mg) was insufficient to elicit the preovulatory response of the hypothalamus and pituitary during the anovulatory season in bison.

Overall, follicular ablation resulted in a shorter interval from treatment to new wave emergence (1.1 day) than either control and estradiol-17β groups. These results agree with those reported in bison (McCorkell *et al.*, 2010) and wapiti (McCorkell *et al.*, 2006) during the anovulatory season where the new follicular emergence was found at 1 and 1.4 days after ablation respectively. In addition, synchrony was less variable (in follicular ablation and estradiol 17β groups as compared to the control group. This supports the hypothesis that follicular ablation and estradiol treatment successfully synchronize follicular waves in bison similar to that reported in cattle, wapiti and bison (Bo *et al.*, 1993; McCorkell *et al.*, 2006; McCorkell *et al.*, 2010).

Treatment with the combination of estradiol plus progesterone has also been associated with regression of the largest follicle and emergence of a new follicular wave (Adams *et al.*, 1994; Bo *et al.*, 1995b). The use of a progestin-releasing ear implant (Synchro-Mate-B, SMB) plus an injection of estradiol-17β was reported as a successful method of ovarian synchronization in cattle (Bo *et al.*, 1994b; Bo *et al.*, 1995b; Bo *et al.*, 1996; Bo *et al*., 2000). Likewise, the administration of estradiol-17β + progesterone in cows that received controlled internal drug

release (CIDR) was efficient to synchronize the new wave emergence at 3.4 days after treatment (Martinez *et al*., 2000). In addition, a single injection of estradiol-17β plus progesterone in sesame oil induced atresia of the dominant follicle and synchronization of follicular wave emergence in camels (Skidmore *et al.*, 2009), llamas (Ratto *et al.*, 2003), and wapiti (McCorkell *et al*., 2006). The synergistic action of these two hormones could be due to the suppressive effect of progesterone on LH pulses which would suppress the growth of LH-dependent follicles enhancing the action of estradiol (Savio *et al.*, 1993; Adams *et al*., 1992a; Bo *et al.*, 1995b). There is no previous report of synchronization of follicular wave emergence in bison by using the combination of estradiol and progesterone. However, first attempts to synchronize estrus in bison during the ovulatory season were done by using SMB and estradiol valerate (Matsuda *et al*., 1996; Othen *et al*., 1999). Estrus was detected in 25%, 55%, and 20% of females on Days 2, 3, and 4 after SMB removal, respectively. However, display of estrus after progestogen withdrawl commonly occurs regardless of whether ovulation ensues. Our study did not involve estrus detection, but documented ovarian follicular dynamics after treatment. Results of the present study showed that estradiol-17β + progesterone treatment induced a new follicular wave at a consistent time relative to treatment (3.3 ± 0.33) in bison. In short, our results support the hypothesis that treatment with estradiol-17β in combination with progesterone may be used effectively to control and synchronize follicular wave development in bison and thus will be useful for ovulation synchronization programs.

In Experiment 2, the interval from treatment to new wave emergence was shorter in follicular ablation group than in estradiol 17β + progesterone or the untreated control groups. In addition, synchrony of follicular wave emergence was less variable in the follicular ablation and estradiol 17β + progesterone groups than in control group. These results are in agreement with

those reported in cattle, camels, and camelids, where the treatment with the combination of steroid hormones or follicular ablation resulted in a synchronous emergence of a new follicular wave (Adams *et al.*, 1994; Bo *et al.*, 1995b, Skidmore *et al.*, 2009; Ratto *et al.*, 2003). Therefore, either estradiol-17β + progesterone treatment or follicular ablation can be used as a means to synchronize follicular wave emergence in wood bison.

The success of ovarian superstimulatory treatments is dependent of the status of follicular waves at the time of initiating treatment (Adams, 1994, Nasser *et al*., 1993). Gonadotropin treatments during the static stage of the wave when a dominant follicle is present resulted in low number of ovulations in cattle (Adams *et al*., 1993). However, the superstimulatory treatment initiated on the day of expected follicle wave emergence resulted in high ovarian response and multiple ovulations in heifers (Adams *et al*., 1993; Adams, 1994). Therefore, synchronization of the emergence of a new follicular wave is an important tool that can be used in bison for further estrus synchronization, fixed-time AI, ovarian superstimulation and superovulation programs.

In summary, results of the present study support the hypothesis that follicular waves can be effectively synchronized in wood bison, and that follicular ablation, estradiol-17β or estradiol-17β + progesterone treatments all shortened and decreased the variability in the interval to wave emergence in bison. Follicular ablation consistently induced an early and very synchronous response and could be used in future studies involving ovarian superstimulation in wood bison.

4.6 Acknowledgements

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5. EFFECT OF GONADOTROPINS ON OVARIAN RESPONSE AND OOCYTE COLLECTION IN WOOD BISON DURING THE ANOVULATORY SEASON

5.1 Abstract

Reclamation of Canada's threatened wood bison herd is complicated by endemic diseases. Eradication of affected herds and repopulation with healthy bison obtained through assisted reproductive biotechnology has been recommended. Accordingly, the goal of this project was to produce disease-free gametes and embryos using assisted reproductive biotechnologies. The specific objective was to compare the ovarian response and oocyte quality in bison given a superstimulatory dose of eCG or pFSH, with or without, a follow-up dose of pLH to induce oocyte maturation during the anovulatory season in a 2 X 2 factorial design. The study was done in two replicates involving a crossover design so that each animal was given the opposite superstimulatory treatment (eCG or pFSH) during successive replicates and randomly chosen for pLH treatment. A 20-day rest period was allowed between replicates. Follicular wave emergence was synchronized by follicular ablation, and 1 day later, bison were assigned randomly to the two superstimulatory treatment groups (n=10/each): i) a single dose of 2500 IU of eCG, and ii) two doses of 200 mg of FSH each. On the day of cumulus-oocyte complex (COC) collection the number of follicles ≥5mm was recorded by transrectal ultrasonography. COC was collected by transvaginal ultrasound-guided follicle aspiration and quality was classified according to morphologic attributes of the ooplasm and number of surrounding cumulus cells (compact, expanded, and denuded). The number of follicles ≥ 5mm (mean ± SEM) was higher ($P < 0.05$) in pFSH- than eCG-treatment groups (14.2 ± 1.41 vs. 8.2 ± 0.67, respectively). COC collection rate did not differ between groups ($P = 0.26$). The proportion of expanded COC was higher ($P < 0.05$) in groups treated with pLH than those without pLH. In conclusion, oocyte collection by

transvaginal ultrasound-guided follicle aspiration from superstimulated bison was feasible and practical. Treatment with pFSH was more effective than eCG in inducing ovarian superstimulation and high quality COC in bison during the anovulatory season, and follow-up treatment with pLH increased the proportion of expanded COC.

5.2 Introduction

Wood bison (*Bison bison athabascae*) are the largest of the wild mammals in North America and probably the most emblematic wild animal of Canada. However, the largest reserve of wood bison in the world, Wood Buffalo National Park in northern Alberta, Canada, remains endemically infected with tuberculosis and brucellosis which hamper population growth and represent a risk of infection to other animals including domestic livestock outside the park (McCormack, 1992; Mitchell and Gates, 2002). In 1990, the Federal Environmental Assessment Review Panel (FEARO) concluded that "eradication of affected herds is the only way to avoid the risk of transmission of these diseases" and recommended repopulating the park with healthy wood bison obtained through reproductive salvage procedures after a reasonable fallow period to ensure that the park is free of latent brucellosis and tuberculosis (Environmental Assessment Panel, 1990).

In vitro embryo production (IVP) is a technology that can be used to help in reclamation of endangered species (Solti *et al*., 2000). Advantages of the IVP as compared to *in vivo* embryo collection in non-domestic animals include: avoiding the problem of timing of ovulation, the possibility of producing more embryos, the ability to make use of diseased or infertile animals, the possibility to salvage genetic material after death, etc. (Loskutoff *et al*., 1995; Holt and Pickard, 1999; Rao *et al*., 2010; Mahesh *et al*., 2011). For conservation purposes, germ plasm

(sperm and oocytes) can be collected from live captive animals (Berg and Asher, 2003; Hermes *et al*., 2009) or be salvaged soon after death (Rao *et al*., 2010). Salvage at the time of death obviates the necessity for animal handling and associated facilities. In bison, successful IVP from slaughtered animals has been reported recently (Thundathil *et al*., 2007). In this study, a high rate of cleavage was found (75%), but the percentage of blastocyst development was very low (7.5%).

In wild animals, development of IVP has been difficult because of the difficulty in obtaining oocytes from abattoir ovaries due to slaughterhouses not always being available. Attempts to collect oocytes from superstimulated live donors by transvaginal ultrasound-guided follicle aspiration (TVFA) have been reported recently in red deer, wapiti, and rhinoceros, with encouraging results (Berg and Asher, 2003; Hermes *et al*., 2009). Ovarian superstimulation enables the collection of a greater number of oocytes per donor. No reports about ovarian superstimulation and oocyte collection by TVFA from live wood bison have been reported.

To develop protocols of superstimulation in bison, it is necessary to understand the reproductive physiology in this species. Follicular and luteal dynamics in bison were recently studied in our laboratory by serial ovarian ultrasound examination (McCorkell *et al*., 2008). In preliminary studies, ovarian synchronization protocols in bison involved a single dose of estradiol 17β, or the combination of estradiol 17β and progesterone, or follicular ablation all of which resulted in synchronous emergence of a new follicular wave (McCorkell *et al*., 2010). Previously, protocols for estrus synchronization and superovulation during the breeding season with modest results have been reported in bison (Dorn *et al.*, 1990, Matsuda *et al.*, 1996; Robison *et al.*, 1998; Othen *et al.*, 1999). However, attempts to perform ovarian superstimulation during the non-breeding season in bison have not been reported.

Ovarian superstimulation in cattle is usually accomplished by administration of gonadotropins such as follicle-stimulating hormone (FSH) and equine chorionic gonadotropin (eCG) (Goulding *et al.,* 1991; Mapletoft *et al*., 2002). While a single injection of eCG induced superstimulation in cattle (Mapletoft *et al*., 1990; Dieleman and Bevers, 1993), treatment with FSH requires twice daily injections for 4 or 5 days to induce a superstimulatory response in cattle (Mapletoft *et al*., 2002). Twice daily injection of FSH necessitates excessive handling, which might adversely affect ovarian response in wild animals such as wood bison (Solti *et al*., 2000; Matsuda *et al.,* 1996). Others have reported successful superstimulation in cattle with single (Bo *et al.,* 1994a) or double (Lovie *et al*., 1994; Alvarez *et al*., 2010) subcutaneous injections of FSH. Reduced numbers of treatments for superstimulation could be used to reduce the handling and stress especially in those species difficult to treat in a frequent basis.

Maturation of oocytes is triggered by the preovulatory LH surge and this process causes expansion of the cumulus cells (Russell and Robker, 2007). *In vivo* matured oocytes have a higher developmental capacity than *in vitro* matured oocytes, suggesting that intrinsic follicular events are necessary to acquire the capacity to fertilize and develop subsequent embryo stages (Dieleman *et al*., 2002; Lanzendorf, 2006). In cattle, the proportion of expanded cumulous oocyte complexes (COC) collected from superstimulated females was increased by the administration of GnRH (Laurincik *et al*., 1993). In seasonal breeders (i.e., ewes), LH levels are normally low during the non-breeding season due to the inhibitory effects of estradiol exerted on the anterior pituitary (Karsch *et al*., 1993). Therefore, the supplementation of exogenous LH in a superstimulatory treatment protocol would likely be necessary to induce *in vivo* maturation of oocytes prior COC collection.

The objectives of this study in wood bison were to compare the ovarian response to superstimulatory treatment with eCG vs. FSH during the anovulatory season, determine the effect of LH treatment after superstimulation on oocyte morphology, and determine the efficiency of oocyte collection via transvaginal ultrasound-guided follicle aspiration.

5.3 Materials and methods

5.3.1. Animals

The study was done during the late anovulatory season (July – August) on twenty mature non-lactating female wood bison (n = 14) and plains bison (n = 6) aged between 4 and 11 years. The bison were maintained on pasture at the Native Hoofstock Centre, University of Saskatchewan, and supplemented with approximately 1 kg/head/day of pelleted feed consisting of alfalfa (50%) and oats (50%) to maintain an average body condition score of 3.5 (scale of 1 to 5; Vervaecke *et al*., 2005). Fresh water was available *ad libitum*. The experimental protocol was approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

5.3.2. Experimental design and treatment groups

A 2x2 factorial design was used to determine the effects of superstimulatory treatment (eCG or FSH) and LH (with or without LH at the end of superstimulation) on ovarian response and cumulus-oocyte complex (COC) collection rate and morphology. The study was done in two replicates involving a crossover design so that each animal was given the opposite superstimulatory treatment (eCG or FSH) during successive replicates and randomly chosen for LH treatment. A 20-day rest period was allowed between replicates.

Follicular wave emergence was synchronized among bison by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter (follicular ablation) as previously described (Bergfelt *et al*., 1994). The procedure was done using a 5.0 MHz convex-array probe (ALOKA SSD 900, Tokyo, Japan) modified for transvaginal use in cattle, and disposable 18 ga x 1 ½" vacutainer needle (BD, Mississauga, Ontario, Canada) attached to a 10 mL syringe by a silicon tubing 60 cm long x 1.14 mm internal diameter (Cole-Palmer, Montreal, Quebec, Canada).

On the day after follicular ablation (expected day of wave emergence; Day 0), bison were assigned randomly to two superstimulatory treatment groups (n=10/group). One group was given a single intramuscular dose of 2500 IU eCG (Pregnecol, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) on Day 0, and the other group was given 200 mg NIH-FSH-P1 (Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) subcutaneously on each of Days 0 and 2. On Day 4, bison in each superstimulation group were divided randomly into two subgroups (n=5 each) and given nothing or 25 mg pLH (Lutropin-V, Bioniche Animal Health) intramuscularly.

Ovarian follicular development was monitored daily from the day of onset of superstimulatory treatment (Day 0) to Day 5 (COC collection) by transrectal ultrasonography using a 7.5 MHz linear-array probe (Aloka SSD 900). Female bison were restrained in a squeeze-chute in the standing position without sedation, as previously described (McCorkell *et al*., 2010). Both ovaries were examined systematically, and images were recorded by carefully sketching the number, size, and relative position of all follicles ≥ 4 mm in diameter on a diagram of the ovary, similar to the method described for cattle (Knopf *et al.*, 1989).

5.3.3. COC collection and evaluation

Collection of COC was done on Day 5 (24 hours after treatment with pLH) by transvaginal ultrasound-guided aspiration of all follicles ≥ 5 mm in diameter using the equipment described above for follicular ablation. Bison were restrained in the hydraulic squeeze and caudal epidural anesthesia was induced with 3 to 5 mL of 2% lidocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada) given at the sacro-coccygeal or first intercoccygeal junction. After washing and disinfecting the vulva with a regular iodine solution, the ultrasound probe equipped with a dorsal-mounted needle guide was placed in the vagina. Manipulation per rectum was done in order to bring the ovaries closer to the transducer and display them on the screen. Using a regulated vacuum pump (flow-rate of 20 mL/min; ref), all follicles ≥ 5 mm were aspirated through the vaginal wall through a disposable 18 ga x 2" short-bevel needle (Misawa Medical Industry Ltd, Edogawa-Ku, Tokyo, Japan) connected via silastic tubing (internal diameter 1.14 mm; Cole Palmer, Montreal, Quebec, Canada) and COC were collected on an ova/embryo filter consisting of a 75 millimicron stainless steel screen (Emcon filter; Agtech, Manhattan, Kansas, USA). The collection medium consisted of Dulbecco's phosphate buffered saline (D-PBS, Sigma, St. Louis, Missouri, USA), 0.3% ET Surfactant (Bioniche Animal Health), and 400 IU/L of heparin (heparin sodium injection USP, Sandoz, Boucherville, Quebec, Canada). Follicular aspirates were rinsed and diluted on the filter using collection medium without surfactant, and then poured into a 90 mm Petri dish. The COC were located, sorted, and morphologically classified using a stereomicroscope (SMZ 1000, Nikon Instrument Inc., Melville, NY, USA) at a magnification of 10X to 40X. The COC were classified according to the number of cumulus cell layers and the appearance of the oocyte cytoplasm. Cumulus cell layers were classified as compact (three or more layers of granulosa cells tightly surrounding the oocyte), expanded (cumulus cells expanded or partially dissociated), denuded or degenerated

(oocyte without cumulus cells or with pyknotic granulosa cells and vacuolated ooplasm) (Ratto *et al*., 2007).

5.3.4 Statistical analyses

Data analysis was carried out using the general linear mixed model (GLMM, SAS version 9.2, Cary, NC, USA). The mixed model accommodated fixed effects (eCG or FSH, with or without LH) and random effects (subject, replicate). Data (i.e. number of follicles ≥ 5mm, diameter of the largest follicle at the time of COC collection, number of follicles aspirated, and number of COC collected) were analyzed and compared by two-way analysis of variance (ANOVA) and Tukey post hoc tests. The COC collection rate and morphology were compared by Chi-square tests, corrected for multiple comparisons. A probability of less than 0.05 was considered statistically significant. Data are presented as mean ± SEM, unless otherwise indicated.

5.4 Results

Data from two animals in the eCG group were excluded from analyses for ovarian response due to the disappearance of all ovarian follicles (spontaneous ovulations) on Day 4 (Day 0 = day of superstimulatory treatment). Two other animals from eCG groups were injured on the day of COC collection (Day 5), data pertinent to ovarian response were taken, but they were not subjected to the transvaginal ultrasound-guided follicle aspiration procedure. Accordingly, injured cows were excluded from the analysis pertaining to number of follicles aspirated, number of COC collected, COC collection rate, and COC classification.

No significant effect of replicate or LH treatment were detected on number of follicles ≥ 5mm, diameter of the largest follicle at time of COC collection, number of follicles aspirated or the number of COC collected. Consequently, these data were pooled to compare superstimulatory treatments (eCG vs. FSH). The mean diameter of the largest follicle at the time of COC collection did not differ between groups (P = 0.53). However, all the other measured parameters were higher (P < 0.05) in cows treated with FSH than in those treated with eCG. Results are presented in Table 5.1.

Table 5.1. Ovarian response and COC collection (mean ± SEM) in bison 5 days after initiating superstimulatory treatment on the day of follicular wave emergence with either eCG or FSH (mean ± SEM).

	Number of follicles ≥ 5 mm	Diameter (mm) largest follicle	Number of follicles aspirated	Number of COC collected
eCG (n=18)	8.2 ± 0.67[a]	11.7 ± 0.48[a]	6.3 ± 0.64[a]	3.8 ± 0.68[a]
FSH (n=20)	14.2 ± 1.41 [b]	12.1 ± 0.37[a]	12.4 ± 1.28[b]	6.7 ± 1.03[b]

[ab] Within columns, values with different superscripts are different (p ≤ 0.05)

Collection rates did not differ (P = 0.26) among groups (eCG or FSH, with or without LH). The proportion of expanded COC did not differ among super stimulation groups treated with LH or the eCG group not treated with LH but all were higher than the FSH group not treated with LH (P < 0.05). In addition, the proportion of expanded COC was higher than compact COC in superstimulation groups treated with LH (P < 0.05), while the proportion of compact COC was higher (P < 0.05) than expanded COC only in the FSH group not treated with LH. The

proportions of compact and expanded COC did not differ in the eCG group not treated with LH. A summary of these results are presented in Table 5.2.

Table 5.2. Recovery rate and morphologic characteristics of cumulus-oocyte complexes (%) collected from 36 female bison after ovarian superstimulation with eCG or FSH followed by nothing or administration of LH.

		eCG with LH (n = 8)	eCG without LH (n = 8)	FSH with LH (n = 10)	FSH without LH (n = 10)
No. of aspirated follicles		52	49	125	122
No. of COC collected		33	27	74	60
COC collection rate	%	63.5	55.1	59.2	49.2
Morphological classification of COC					
Compact	%	24.2 $^{a, x}$	48.1^{a,x}	35.1^{a,x}	78.3^{b,x}
Expanded	%	60.6 $^{a, y}$	37.0^{a,x}	54.1^{a,y}	15.0^{b,y}
Denuded	%	15.2 ^{z}	14.8 ^{y}	10.7 ^{z}	6.7 ^{z}

^{a,b} Values within rows with different superscripts are different ($P < 0.05$)
^{x,y,z} Values within columns with different superscripts are different ($P < 0.05$)

5.5 Discussion

Results of the present study show that successful ovarian superstimulation and oocyte collection can be accomplished in female bison during the anovulatory season. This is an important approach in the development of reproductive techniques for the production of embryos at a time other than the breeding season.

In our study, twenty bison were successfully synchronized and superstimulated with both gonadotropins (FSH or eCG) inducing multiple development of follicles ≥ 5 mm during the non-breeding season. Seasonally anestrous ewes have been similarly treated with FSH or eCG which induced ovarian superstimulatory response (Azawi and Al-Mola, 2010). Therefore, it could be suggested that the sensitizing effect of progesterone seems not to be a prerequisite for obtaining a high ovarian response in superstimulated bison during the non-breeding season.

In average our results showed that the number of follicles ≥ 5mm at the day of COC collection was higher in females treated with FSH than those treated with eCG. There are several studies that show similar results in cycling cattle (Sendag *et al*., 2008) and in sheep during the breeding season (Walker *et al*., 1986) and the non breeding season (Azawi and Al-Mola, 2010) suggesting that FSH is more effective than eCG in inducing superstimulation. Therefore, results from our study as well as from others suggest that FSH is more efficacious as a superstimulatory treatment than eCG and it should be used in further programs of superstimulation in bison.

It has been reported previously that eCG induces a lower superovulatory response than FSH in cycling cattle (Monniaux *et al*., 1983). Even though long half-life of the eCG was identified as responsible for the low superovulatory response (Mapletoft *et al*., 2002; Goulding *et al*., 1996), for the ovarian superstimulation and COC aspiration, the long half-life of eCG should not be a problem and perhaps we are dealing with an issue of dose and biological activity (Newcomb *et al*., 1979; Saumande and Chupin, 1986). Conversely, several studies have shown that a single dose of eCG (1500 to 3000 IU) induces superstimulatory ovarian response in cattle and its effect is enhanced with the incorporation of an antibody to eCG in the treatment protocol which results in a highly satisfactory superovulatory response (Dieleman *et al*., 1993; Gonzales *et al*., 1994).

These findings contrast with our results and we can speculate that the effect of eCG would be different according to the species treated.

Likewise, the short half-life of the FSH (around 5 hours) necessitates that it be administered in multiple doses given twice a day for 4 to 5 days (Monniaux *et al*., 1983). In order to reduce handling associated with treatment, FSH it was administered as a bolus subcutaneously behind the shoulder to slow absorption into the circulation due to the fat tissue in the area of injection (Bo *et al*., 1994a; Mapletoft *et al*., 2002). Several studies have been conducted in cattle whereby a single (Bo *et al*., 1994a) or two (Lovie *et al*., 1994; Alvarez *et al*., 2010) subcutaneous doses of FSH resulted in an ovarian response comparable to those reported by using the traditional multiple dose treatment protocol. However, results were highly dependent on the amount of subcutaneous fat at the site of injection. In our study, we obtained a superstimulatory ovarian response at least equivalent to that reported previously utilizing multiple injections of FSH in bison during the breeding season (Othen *et al*., 1998; Matsuda *et al*., 1996).

Transvaginal ultrasound-guided follicle aspiration (TVFA) was first developed in cattle to permit collection of oocytes for IVP from live cattle (Pieterse *et al*., 1988). However, this technique has been adapted recently for use in wild animals such red deer, wapiti and rhinoceros (Berg and Asher, 2003; Hermes *et al*., 2009). In bison, TVFA has been reported as a method of synchronization of a new follicular wave emergence (McCorkell *et al*., 2010), but the use of this technique for oocyte collection has not been previously reported. To the best of our knowledge, this study is the first that reports successful collection of oocytes by TVFA in bison during the anovulatory season.

Overall, 348 follicles were aspirated by TVFA and 194 COC were collected from 16 bison cows (on two occasions) which correspond to a COC collection rate of 56%. This is comparable

to that achieved in cattle (Pieterse *et al*., 1991) and indicates promise for this technique in the application of IVP technology in bison. In the present study we investigated the effect of the administration of LH on the COC collection rate in each superstimulatory treatment. We hypothesized that LH would cause expansion of the cumulus cells and weaken their fixation to the follicular wall (Mattioli and Barboni, 2000; Russell and Robker, 2007), which would be expected to improve oocyte collection efficiency. Although we found a higher percentage of collection rate in the eCG and FSH treatment groups treated with LH (63.5 and 59.2%, respectively) as compared to those not treated with LH (55.1 and 49.2%, respectively), there were no statistical differences among treatments. Our results agree with those reported by Chaubal *et al*. (2006), but contrast with Bordignon *et al*. (1997) who reported higher oocyte collection rate when superstimulated cows were treated with GnRH in cattle. These differences can be due to species used or the type of hormone administrated (pLH vs GnRH).

In our study we decided to investigate the use of exogenous LH to induce *in vivo* maturation of oocytes as represented by the presence of expanded COC. As we expected, the proportion of expanded COC was higher than compact oocytes in eCG and FSH treated groups that were also treated with LH while the proportions of compact COC were higher than expanded oocytes in the FSH group that was not treated with LH. However, there were no differences between the proportion of compact and expanded oocytes in the eCG group that was not treated with LH. We speculated that the LH activity of the eCG had an effect on medium and large follicles inducing early activation and cumulus expansion which may not desirable. Therefore, expanded oocytes obtained from LH treated animals may be considered as ovulated oocytes, and they could be used directly for *in vitro* fertilization in the same way as has been used in other species such as cattle (Sirard and Blondin, 1996) and camels (Khatir *et al*., 2007).

In summary, treatment with FSH was more effective than eCG to induce ovarian superstimulation in bison during the anovulatory season. Transvaginal ultrasound-guided follicular aspiration was a practical and feasible technique that could be performed in bison in order to collect a substantial number of oocytes from live animals. Including LH at the end of the superstimulation treatment protocol increased the proportion of expanded COC obtained from seasonally anestrous superstimulated bison providing new alternatives for the development of *in vitro* embryo production in this species.

5.6 Acknowledgements

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6. Ovarian superstimulation and oocyte collection in wood bison (*Bison bison athabascae*) during the ovulatory season.

6.1 Abstract

As part of an overall goal to preserve the threatened population of wood bison, the objective of this study was to establish an effective superstimulatory protocol and obtain oocytes by transvaginal ultrasound-guided follicular aspiration. Two experiments were done involving 22 wood bison during the breeding season (September to December). In Experiment 1, the bison were given a luteolytic dose of prostaglandin (Day 0) and underwent follicular ablation (Day 8) to induce ovarian synchrony. Synchronized bison were assigned randomly to two groups (n=11/group) and given 1) 200 mg pFSH diluted in saline subcutaneously on Days 9 and 11, or 2) 200 mg pFSH diluted in a proprietary slow release formulation (SRF) intramuscularly on Days 9 and 11. Prostaglandin was given on Day 11 followed by 25 mg pLH on Day 13. Oocytes were collected by transvaginal ultrasound-guided aspiration of follicles ≥ 5 mm 24 hours after pLH. In Experiment 2, bison were synchronized as Experiment 1 and assigned randomly to two groups (n=11/group) and given: 1) a single dose of 2500 IU eCG intramuscularly on Day 9, or 2) 200 mg pFSH subcutaneously on Days 9 and 11. Prostaglandin was given on Day 11, and pLH (25 mg) was given on Day 13. Oocyte collection was done as described in Experiment 1. Cumulus-oocyte-complexes (COC) were classified according to morphological characteristics. In Experiment 1, the pFSH-Saline and pFSH-SRF groups were similar in the mean (± SEM) number of follicles ≥ 5 mm on Day14 (12.4 ± 1.49 vs 13.8 ± 1.24, respectively) and the number of COC collected (6.5 ± 1.13 vs 6.3 ± 0.96, respectively). In Experiment 2, more follicles ≥ 5mm on Day 14 were detected in bison treated with pFSH vs eCG (12.2 ± 1.73 vs 5.8 ± 0.52; $P < 0.05$), and more COC were collected from pFSH-treated animals (7.2 ± 1.41 vs 3.4 ± 0.62; $P <$

0.05). The proportion of COC collected per follicle aspirated and the percentage of compact, expanded, and denuded oocytes did not differ between groups in either Experiment 1 or Experiment 2. In summary, a 2-dose regime of pFSH, diluted in either saline and injected subcutaneously, or a slow-release formulation and injected intramuscularly, induced a similar ovarian response in wood bison, while bison given a single-dose of 2500 IU eCG had a significantly lower ovarian response. Overall, a COC were collected from 55% of follicles following transvaginal ultrasound-guided needle aspiration in wood bison.

6.2 Introduction

Wood bison (*Bison bison athabascae*) herds in and around Wood Buffalo National Park remain endemically infected with tuberculosis and brucellosis (McCormack, 1992). These diseases threaten to infect neighboring healthy wood bison herds, domestic cattle and bison ranches (Mitchell and Gates, 2002). Thus, the Federal Environmental Assessment Review Panel (FEARO) recommended eradication of diseased wood bison herds to avoid the transmission of these diseases to domestic cattle and uninfected wood bison. The panel further recommended that the area should be repopulated with healthy wood bison obtained through genetic salvage operations (Environmental Assessment Panel, 1990).

The use of assisted reproductive technologies (ART) in wildlife has not been developed as in domestic animals. In bison, attempts to develop ovarian synchronization and superovulation protocols have been reported with modest results (Dorn *et al*., 1990; Othen *et al*., 1999; Matsuda *et al*., 1996). These studies were conducted following protocols that have been based on results of studies carried out in cattle. Unfortunately, results in bison have been affected by problems similar to cattle, such as variable ovarian response and abnormal follicle and oocyte development

(Greve *et al*., 1995; Mapletoft *et al*., 2002). Lack of information in bison on follicle dynamics, follicular population, and effects of handling stress do not allow us to use ART. Therefore, to deal with these potential problems and develop ovarian superstimulation protocols in bison we needed to use information that had been acquired in cattle.

Early studies in cattle revealed the influence of follicular wave status on the superstimulatory response (Adams, 1994; Adams *et al*., 2008). Results demonstrated that the response was optimal when treatment was initiated at the time of wave emergence or before selection of the dominant follicle (Nasser *et al*., 1993). Administration of estradiol in combination with progesterone in cattle resulted in synchronous emergence of the next follicular wave, on average, 4 days later, regardless of the stage of the cycle at the time of treatment (Bo *et al*., 1995a; Bo *et al*., 1995b; Bo *et al*., 1994b). Transvaginal ultrasound-guided ablation of follicles ≥5 mm has been developed as an alternative method to synchronize follicular wave emergence, and resulted in synchronous emergence of a new follicular wave, on average, 1 day later (Bergfelt *et al*., 1994). These methods of synchronization have been used to develop ovarian superstimulation protocols in cattle (Bergfelt *et al*., 1997; Baracaldo *et al*., 2000; Bo *et al*., 1996).

The most widely used hormones to induce superstimulation in cattle are follicle stimulating hormone (FSH) derived from pituitary extracts and equine chorionic gonadotropin (eCG) (Goulding *et al*., 1991). Successful superovulatory ovarian responses have been obtained with a single dose of eCG followed by a dose of anti eCG in cattle (Dieleman and Bevers, 1993; Mapletoft *et al*., 1990). Likewise, FSH given in twice daily constant or decreasing doses for four days has shown to induce ovarian superstimulation in cattle (Monniaux *et al*., 1983; Gonzalez *et al*., 1990). Further, a single and double subcutaneous injection of FSH in cattle have been shown to induce ovarian superstimulation and results were equivalent to those obtained in the traditional

twice daily doses of FSH (Bo *et al*., 1994a; Lovie *et al*., 1994; Alvarez *et al*., 2010). However, response to the single subcutaneous injection of FSH in saline was dependent on sufficient subcutaneous adipose tissue to slow the absorption of FSH. Therefore, single intramuscular injection protocols involving the use of a proprietary slow-release formulation have been developed (Bo *et al*., 2010b). Because of the stress associated with twice daily injections, less-frequent superstimulatory protocols were considered more useful in wild species (e.g. bison) that are difficult to handle on a frequent basis.

Ultrasound-guided transvaginal follicular aspiration has been used to provide oocytes for *in vitro* embryo production in both non-stimulated and superstimulated cattle and alpaca (Brogliatti *et al*., 2000; Galli *et al*., 2001). Cumulus-oocyte-complexes (COC) must undergo maturation before *in vitro* fertilization. Treatment with GnRH before follicular aspiration has been used in cattle to induce *in vivo* oocyte maturation and improve COC collection rate (Laurincik *et al*., 1993; Bordignon *et al*., 1997). Transvaginal oocyte collection and the effects of hormone treatment on COC maturation in bison during the breeding season have not been reported.

Recent studies involving serial ultrasonographic examination of bison have provided an understanding of ovarian follicular and luteal dynamics in this species (McCorkell, 2008) and have permitted the development of effective ovarian synchronization protocols (McCorkell *et al*., 2010). The objective of the present study was to develop an effective ovarian superstimulation protocol for the purposes of *in vitro* embryo production in bison. The ovarian response and the morphologic attributes of COC collected by transvaginal ultrasound-guided follicle aspiration were compared among bison treated with a short- vs long-acting preparation of pFSH (Experiment 1), and treated with pFSH vs eCG (Experiment 2).

6.3 Materials and methods

6.3.1 Animals

Female bison (*Bison bison*; n=22) between 4 and 11 years of age were used during September to December (ovulatory season). The bison were non-lactating, with an average body condition score of 3.5 (scale of 1 – 5) (Vervaecke *et al*., 2005) and were cyclic at the beginning of the study as indicated by ultrasonographic detection of a corpus luteum. They were placed on pasture with free access to supplemental hay and fresh water at the Native Hoofstock Centre, University of Saskatchewan (52º08'N, 106º38'W). The bison were handled according to a protocol approved by the University of Saskatchewan Committee on Animal Care and Supply under the guidelines of the Canadian Council on Animal Care.

6.3.2 Experiment 1

Ovarian follicular and luteal function were synchronized among bison by giving a luteolytic dose of prostaglandin (25 mg of Dinoprost, Lutalyse, Pfizer, NY, USA; Day 0) followed by transvaginal ultrasound-guided aspiration of all follicles ≥5 mm in diameter (follicular ablation) on Day 8, as previously reported in cattle and bison (Bergfelt *et al*., 1994; McCorkell *et al*., 2010). Follicular ablation was done using a 5 MHz intravaginal probe (ALOKA SSD-900, Tokyo, Japan) equipped with a disposable 18 ga x 1½" vacutainer needle (BD, Mississauga, Ontario, Canada) attached to a 10 ml syringe by a 60 cm long x 1.14 mm internal diameter silicone tubing (Cole-Palmer, Montreal, QC, Canada).

Bison were assigned randomly to two groups (n=11/group) and given either pFSH in saline (Folltropin-V, Bioniche Animal Health Canada Inc., Bellville, Ontario, Canada) or a long-acting preparation of pFSH in a slow-release formulation (50% SRF, Bioniche Animal Health). For

both groups, a total dose of 400 mg NIH-FSH-P1 of pFSH was divided into two doses (200 mg each) and given subcutaneously behind the shoulder (FSH-saline) or intramuscularly in the neck (FSH-SRF) on Days 9 and 11. A luteolytic dose of prostaglandin (25 mg of Dinoprost) was given im on Day 11 to suppress negative effect of progesterone on LH. Then, 25 mg Armour standard LH (Lutropin-V, Bioniche Animal Health) IM on Day 13 to evaluate its effect on *in vivo* maturation and to increase cumulus-oocyte complexes (COC) collection rate. Ovarian follicular development was monitored daily from Day 8 (follicular ablation) until Day 14 (COC collection) by transrectal ultrasonography using a 7.5 MHz probe (ALOKA SSD-900, Tokyo, Japan), as previously described (McCorkell, 2008). Treatments were scheduled so that collection of COC was done on 4 or 5 animals per day.

The COC were collected on Day 14 by transvaginal ultrasound-guided aspiration of all follicles ≥7 mm in diameter, as described for cattle (Hashimoto *et al*., 1998; Brogliatti *et al*., 1996). Briefly, bison were restrained in a squeeze chute and caudal epidural anesthesia was induced by injection of 3 to 5 ml of 2% lidocaine hydrochloride (Bimeda-MTC, Animal Health Inc., Cambridge, ON, Canada) between the first two caudal vertebrae. The vulva was washed with detergent and disinfectant three times before placing the transvaginal probe into the vagina. The COC were aspirated and collected through a disposable 18 ga x 2" short-bevel needle (Misawa Medical Industry Ltd, Edogawa-Ku, Tokyo, Japan) connected via silicone tubing (80 cm x 1.14 mm ID, Cole-Palmer, Montreal, Canada) to an Emcon filter (75 µm, Agtech, Manhattan, Kansas, USA) using a regulated vacuum pump set at a flow rate of 20 ml/min. The filter contained collection medium consisting of Dulbecco's phosphate buffered saline (D-PBS, Sigma, St. Louis, Missouri, USA), 0.3% ET Surfactant (Bioniche Animal Health), and 400 IU/L of heparin (sodium heparin injection USP, Sandoz, Boucherville, QC, Canada). The aspirated

fluid was diluted and washed by passing collection medium through the filter. The filter was washed with collection medium (without ET surfactant) and the contents were poured into 90 mm Petri dishes.

The COC were identified using a stereomicroscope (SMZ 1000, Nikon Instrument Inc., Melville, NY, USA) at a magnification of 10X - 40X and were morphologically classified according to characteristics of the surrounding cumulus cells (Torner *et al*., 1998; Ratto *et al*., 2007) as compact: oocyte enclosed in one or more layers of cumulus investment; expanded: cumulus cells expanded or partially dissociated; denuded: oocyte without cumulus cells and/or has a degenerated cytoplasm.

6.3.3 Experiment 2

After an interval of ≥50 days from the end of Experiment 1 (i.e., after COC collection), the same bison were re-synchronized (as described in Experiment 1) and allocated randomly into two groups (n=11/group) and given either pFSH (Folltropin-V) or eCG (Pregnecol, Bioniche Animal Health). Folltropin-V in saline was given subcutaneously as in Experiment 1 (200 mg on Days 9 and 11), and a single dose of eCG (2500 IU) was given intramuscularly in the neck on Day 9. As in Experiment 1, a luteolytic dose of prostaglandin (25 mg of Dinoprost IM) was given on Day 11 followed by 25 mg Armour standard pLH (Lutropin-V) intramuscularly on Day 13. Ovarian follicular development was monitored ultrasonically as in Experiment 1, and COC were collected and evaluated on Day 14, as described in Experiment 1.

6.3.4 Statistical analysis

Single-point measurements (i.e., number of follicles ≥5mm, diameter of the largest follicle at the time of COC collection, number of follicles aspirated, and number of COC collected) were analyzed by Student's t-test to determine differences between groups. The percentage of COC collected and COC morphology were compared between groups by Chi square. SAS software package version 9.2 (SAS Institute Inc., Cary, NC, USA) were for statistical analysis. Values were tested for normality with Shapiro-Wilk test before comparing treatments ($P \leq 0.05$).

6.4 Results

6.4.1 Experiment 1. Effect of pFSH saline vs SRF

No differences were detected between groups in the number of follicles ≥ 5 mm, the diameter of the largest follicle, the number of follicles aspirated, at the time of COC collection (24 hours after pLH treatment), or the number of COC collected (Table 6.1). Similarly, no differences were detected between groups in the COC collection rate or in the proportion of COC in the respective morphologic categories. However, a higher proportion ($P<0.05$) of expanded COC than compact COC were found in both treatment groups (Table 6.2).

Table 6.1 Effect of short-acting (FSH-saline) and long-acting (FSH-SRF) preparations on ovarian superstimulatory response and COC collection in wood bison (mean ±SEM).

	Number of follicles ≥5mm	Diameter (mm) largest follicle	Number of follicles aspirated	Number of COC collected
FSH-Saline (n=11)	12.4 ± 1.49	10.5 ± 0.59	11.0 ± 1.49	6.5 ± 1.13
FSH-SRF (n=11)	13.8 ± 1.24	11.5 ± 0.76	11.4 ± 1.49	6.3 ± 0.96

No significant differences between groups for any end point

Table 6.2 Collection rate and morphology of cumulus-oocyte-complexes (COC) following pLH treatment of wood bison superstimulated with pFSH diluted in either saline or a slow-release formula (SRF).

	Follicles aspirated	COC collected	Collection rate (%)	COC morphology (%)		
				Compact	Expanded	Denuded
FSH-Saline (n=11)	121	72	59.5	17[a]	79[b]	4[a]
FSH-SRF (n=11)	125	69	52.2	16[a]	77[b]	7[a]

[ab]Values within rows with different superscripts are different ($p \leq 0.05$)

6.4.2 Experiment 2. Effect of eCG vs pFSH

At the time of COC collection, there was no difference between groups in the diameter of the largest follicle, but the number of follicles ≥ 5mm ($P<0.05$) and the number of COC collected ($p<0.01$) were higher in bison treated with pFSH in saline than those treated with eCG (Table 6.3). The COC collection rate (eCG: 67.3% and FSH: 60.8%) and the proportion of COC in the respective morphologic categories did not differ between groups. As in Experiment I, the proportion of expanded COC was higher ($P<0.05$) than compact COC for both treatment groups (Table 6.4).

Table 6.3 Ovarian response to superstimulatory treatments with pFSH or eCG in wood bison (mean ±SEM).

	Number of follicles ≥5mm	Diameter (mm) largest follicle	Number of Follicle aspirated	Number of COC collected
eCG (n=11)	5.8 ± 0.52[a]	9.9 ± 0.59	5.6 ± 0.47[a]	3.4 ± 0.62[a]
FSH (n=11)	12.2 ± 1.73 [b]	9.8 ± 0.48	11.2 ± 1.79[b]	7.2 ± 1.41[b]

[ab]Values within columns with different superscripts are different ($P \leq 0.05$)

Table 6.4 Collection rate and morphology of cumulus-oocyte-complexes (COC) in wood bison given pLH treatment following superstimulation with pFSH in saline or eCG.

	Follicles aspirated	COC collected	Collection rate (%)	COC morphology (%)		
				Compact	Expanded	Denuded
eCG (n=11)	62	37	67.3	6[a]	85[b]	9[a]
pFSH (n=11)	123	79	60.8	11[a]	73[b]	16[a]

[ab]Values within rows with different superscripts are different ($P \leq 0.05$)

6.5 Discussion

Altogether, twenty-two normal cyclic female bison, between 4 and 11 years of age, were successfully superstimulated twice (in two experiments) during the breeding season. In addition, follicular ablation was used to successfully synchronize follicular waves for superstimulation. Overall, 431 follicles were transvaginally aspirated and 237 COC were collected which corresponds to a COC collection rate of 55%. Similar result was obtained by Kruip *et al*. (1994) who found 55% of COC recovery rate in cattle. However, it contrasts with Chaubal *et al.* (2007) who reported in cattle a 60 – 70% of COC recovery rate. Distinct vacuum pump pressure (90 mmHg vs 70 mmHg) used and technical skills could be involved in these differences (Ward *et al*., 2000). To our knowledge, this is the first time that successful oocyte collection by transvaginal aspiration has been reported in wood bison.

Previous studies in cattle supported the hypothesis that ovarian superstimulation should be initiated at the time of follicular wave emergence to avoid the suppressive effect of the dominant follicle (Adams, 1994; Nasser *et al*., 1993; Adams *et al*., 1994). Small follicles of the new wave require FSH to continue their growth avoiding selection and subsequent subordinate follicle atresia (Monniaux *et al*., 1983). Collapse of dominant follicle performed by follicular ablation

has been reported in cattle as an efficient method to synchronize follicular wave emergence (Bergfelt *et al*., 1994). In bison it was also shown that transvaginal ultrasound-guide follicular aspiration of the largest follicles (≥ 5 mm) will synchronize a new follicular wave 1 day after follicular ablation and with higher degree of synchrony than treatments with steroid hormones (McCorkell *et al*., 2010). Likewise, transvaginal follicular ablation has also been reported to be an effective method to induce the synchronization of a new follicular wave for the purposes of ovarian superstimulation in cattle (Bergfelt *et al*., 1997; Baracaldo *et al*., 2000). The present experiments show that the same applies to bison.

A treatment protocol of multiple doses of exogenous FSH has been used extensively for ovarian superstimulation in cattle (Monniaux *et al*., 1983; Roover *et al*., 2005). Nevertheless, the administration of multiple doses of FSH is not practical for non-domestic animals because of the stress associated with animal handling. Protocols of a single and two subcutaneous doses of FSH in saline were previously reported in cattle which induced an ovarian response equivalent to those treated with the traditional multiple doses (Bo *et al*., 1994a; Lovie *et al*., 1994; Alvarez *et al*., 2010). However, the efficacy of the protocol was dependent on there being sufficient subcutaneous adipose tissue so as to delay the absorption of FSH in saline. This is the reason for the development of a slow-release formulation that would delay absorption of FSH, even following administration intramuscularly. In Experiment 1, it was shown that two subcutaneous doses of FSH in saline behind the shoulder caused satisfactory superstimulation (12.4 ± 1.49 follicles per donor) suggesting that these bison had sufficient subcutaneous adipose tissue (BCS of 3.5 out of 5) for this approach to superstimulation. Similarly, results of the FSH-SRF group in Experiment 1 (13.8 ± 1.24 follicles per donor) are in agreement with previous results in which a single intramuscular injection of FSH in 100% SRF (Bo *et al*., 2010a; Tribulo *et al*., 2010) or

two intramuscular injections of FSH in 50 or 25% SRF (Bo *et al*., 2010a) induced a superovulatory response comparable to twice daily intramuscular injections of FSH in cattle. On the other hand, a single intramuscular injection or split-single intramuscular injection of FSH in SRF have been shown to be efficacious in inducing superovulation or superstimulation of oocyte recovery in goats (Baldassarre *et al.*, 2010) and a single intramuscular injection of FSH in 25% SRF was shown to be efficacious in the induction of superstimulation for oocyte recovery in cattle (Ongaratto *et al*., 2010). Both treatments resulting in a superstimulatory response comparable with those reported in cattle using multiple intramuscular injections of FSH in saline.

Results from Experiment 1 showed that there were no differences between groups for any end point evaluated (Table 6.1) and the use of short-acting FSH (FSH-Saline) as a double subcutaneous injections or long-acting FSH (FSH-SRF) given in two intramuscular doses successfully induced ovarian superstimulation for oocyte recovery in bison. Therefore, we can suggest that these protocols can be considering important tools to minimize handling and stress in wild animals subjected to superstimulatory treatments.

Based on results of Experiment 1, we decided to compare the two-dose subcutaneous protocol of FSH in saline with a single intramuscular injection of eCG in the induction of superstimulation in bison (Experiment 2). Again, the double subcutaneous doses of FSH successfully induced multiple follicular development in bison and confirmed our results obtained in Experiment 1. On the contrary, the use of a single intramuscular injection of 2500 IU of eCG in Experiment 2 induced a significantly lower superstimulatory response. Similar results were found by Sendag *et al*. (2008) who reported a lower superstimulatory ovarian response following eCG treatment as compared to FSH in cattle. Although eCG has the advantage to require just a single dose, its long circulating half-life (3-5 days) seems to be a considerable problem for

superstimulatory treatments due to the induction of persistent follicles or early multiple ovulation (Monniaux *et al*., 1983; Mapletoft *et al*., 2002). The prolonged half-life of eCG has been neutralized with the use of eCG antibodies in cattle with successful superovulation (Dieleman *et al*., 1993), however eCG antibodies are not available commercially and in any case, would represent more handling and cost that may not be feasible in bison.

In Experiment 2, the number of follicles at the time of COC collection (24 hours after pLH treatment) was higher in pFSH group (12.2 ± 1.73) than the eCG group (5.8 ± 0.52). These results agree with those reported in cattle where FSH treatment induced a higher superstimulatory ovarian response than eCG treatment (Goulding *et al*., 1991, Sendag *et al*., 2008). In bison, Othen *et al*. (1999) reported low number of follicles and corpora lutea after treatment with a single dose of eCG. They also reported unsuccessful ovarian superstimulation following a single intramuscular dose of FSH which is not surprising. Bo *et al*. (1994a) reported that hastened absorption of FSH, e.g., intramuscular vs. subcutaneous injection, low body condition score with subcutaneous injection and more recently (Bo *et al*., 2010a) intramuscular injection with FSH in saline vs. SRF, would affect superovulatory response. These differences could be due to the route of administration and the half-life of the FSH. The half-life of the FSH is around 5 hours (Monniaux *et al.*, 1983) and in protocols which use intramuscular injections, it is necessary to use the traditional multiple dose to maintain circulating blood levels for superstimulation (Monniaux *et al*., 1983; Gonzalez *et al*., 1990). Dorn *et al.* (1990) reported the use of the traditional multiple dose of FSH obtaining a high ovarian response in bison. Therefore, FSH seems to be the hormone of choice to use in superstimulatory protocols in bison.

The number of follicle aspirated and COC collected were also higher following FSH as opposed to eCG treatment (Experiment 2). This was due to more follicles available in each

ovary; the more follicles available for aspiration, the more likely the collection of oocytes. This is supported by Goodhand *et al.* (2000) who found that the administration of FSH improved the number of follicles available for aspiration. Likewise, the mean number of recovered oocytes per session was consistent with the mean number of visualized and aspirated follicles in bison treated with FSH. These results are comparable with those reported by Perez *et al.* (2000) in cattle. In addition, the relationship between the number of follicles aspirated and the number of oocyte retrieved in bison agrees with Durocher *et al.* (2006) who found a high association between the number of follicles aspirated and number of oocytes collected in cattle.

Overall, no significant differences were found when COC collection rate and percentage of COC morphology were compared between groups in each experiment. The purpose of giving pLH 24 hours before oocyte collection was to stimulate the maturation of oocytes *in vivo* which would be represented by the number of expanded COC (Laurincik *et al*., 1993; Bordignon *et al*., 1997). The high rate of expanded COC found in our two experiments (FSH-saline: 79% vs. FSH-SRF: 77% and eCG: 85% vs FSH: 73%) suggest that our protocol provides for matured oocytes when pLH is given 24 hours before COC collection. Further studies will be necessary to determine whether these expanded COC can be used for *in vitro* fertilization without *in vitro* maturation.

In conclusion, this study supports our hypothesis that ovarian synchronization and superstimulation are possible in wood bison. Due to its threatened status, assisted reproductive technologies are an important alternative to preserve this population. It was found that treatments with pFSH given intramuscularly in SRF, or subcutaneously in saline are effective in the induction of superstimulation in wood bison, while eCG at the dose tested resulted a lower superstimulatory ovarian response. In addition, our results document successful collection of

viable oocytes from superstimulated wood bison via transvaginal ultrasound-guided follicle aspiration, which would be useful for *in vitro* embryo production protocols. Lastly, successful assisted reproductive technologies are feasible and practical techniques applicable in wood bison.

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7. GENERAL DICUSSION

Wood buffalo National Park (WBNP) located in the extreme north of Alberta that overlapping into the Northwest Territories contains the world's largest genetically diverse population of wood bison. However, some bison herds in the park are infected with two cattle diseases (Brucellosis and Tuberculosis) and threaten to infect healthy bison herds in and around the park (Gates *et al*., 2001; Mitchell and Gates, 2002). Therefore, the Federal Environmental Assessment Review Office recommended in 1990 the eradication of infected bison and repopulation of the area with healthy bison obtained through salvage techniques (Environmental assessment panel, 1990). For that reason, the purpose of this thesis was to develop effective methods of ovarian synchronization and superstimulation in wood bison which would allow us to collect oocytes that could be used in *in vitro* embryo production programs. With this in mind, a genetic resource bank for the threatened wood bison could be established to prevent loss of genetic diversity that would occur after the eradication of infected bison from WBNP.

Two studies were carried out to develop protocols of synchronization, superstimulation and oocytes collection in wood bison. For the synchronization study, two experiments were done during the anovulatory season. For superstimulation and oocyte collection, we carried out one experiment during the anovulatory season and two experiments during the ovulatory season.

7.1 Synchronization of follicular wave development in wood bison

Synchronization of follicular wave emergence would be an important tool for improving the utilization of assisted reproductive technologies in wildlife (Solti *et al.* 2000; Wildt 1992). Synchronization methods developed in cattle have been successfully applied to other wild bovid species such as gaur and banteng (Comizzoli *et al*., 2000; Pukazhenthi and Wildt, 2004).

Therefore, the use of cattle as model to develop ovarian synchronization methods for bison is consistent with this idea. The most effective approaches for synchronization of follicular waves in cattle are physical or hormonal methods that seek to eliminate the inhibitory effect of the dominant follicle on FSH release and the emergence of a new follicular wave in a predictable interval of time after treatment (Adams, 1994; Bo *et al*., 1995b). In the present thesis, follicular ablation and steroid treatments with estradiol-17β or estradiol 17β + progesterone were investigated for their usefulness for the synchronization of follicular development in bison.

The follicular ablation procedure consisted of an ultrasound-guided aspiration of all follicles ≥ 5 mm in diameter as described by Bergfelt *et al.* (1994). The aim of this method is to remove the suppressive effect of the dominant follicle on development of small antral follicles (Bergfelt *et al*., 1997). The dominant follicle secretes estradiol and inhibin which have a negative feedback on FSH secretion (Adams *et al*., 1993). When the largest follicle is removed, this negative effect is lost, FSH surges and a new follicular wave rapidly emerges (Bergfelt *et al.*, 1994; Bo *et al.*, 1995b). Results of our synchronization study in wood bison showed that follicular ablation shortened the interval to emergence of the new follicular wave to 1.1 days (Experiment 1) and 1.2 days (Experiment 2). However, what is most important is that the variability was very tight demonstrating a high degree of synchrony which could be a useful tool for synchronization purposes. Results of the present study are in agreement with those of McCorkell *et al*. (2010) who reported that follicular wave emergence occurred 1 day after follicular ablation in bison during the anovulatory season. Therefore, it should be possible to use follicular ablation as a means to synchronize wave emergence for superstimulatory protocols in wood bison in the same way that is used in cattle (Bergfelt *et al*., 1997). It is also noteworthy that there is no apparent difference in the use of follicular ablation between seasonally anestrus

and cycling bison as demonstrated in studies done in non-cycling and cycling cattle (Brogliatti *et al*., 1997; Bergfelt *et al.*, 1994).

We also examined the effect of the administration of 2 mg of estradiol-17β which induced the regression of the largest follicle and emergence of a new follicular wave 3.1 days after treatment. This result is in agreement with that previously reported in cattle (Bo *et al.*, 1993) where new follicular wave emergence was induced after estradiol treatment. The action of the estradiol in synchronizing follicular wave emergence is not well understood, but it was found that estradiol treatment was associated with decreased levels of FSH resulting in suppression of growth of FSH-dependent (small antral) follicles (Adams, 1999). It would appear that following decreasing levels of estradiol, FSH surges and leads to follicle wave emergence (Adams *et al*., 1992a; Martinez *et al*., 2000; Colazo *et al*., 2003). It has been suggested that apoptosis can be the most important factor in the induction of follicular atresia; however, gonadotropins would play a critical role in preventing apoptosis in granulosa cells; FSH is considered one of the main follicle survival factors (Yang and Rajahamendran, 2000a; Yang and Rajahamendran, 2000b). Therefore, we can speculate that changes in the secretion of FSH and/or LH due to estradiol treatments that were previously reported (Bo *et al*., 1993, Bo *et al*., 1994b) may also be related to the atresia of larger follicles in domestic animals. In this regard, it was also reported that in the ewe, a seasonal breeder, photoperiod governs the response of the hypothalamo-pituitary axis to the negative feedback action of estradiol (Karsch *et al.*, 1993). In fact, estradiol is a potent inhibitor of gonadotropin secretion, exerting its action specifically on LH pulse frequency during long days in ewes (Thiery and Malpaux, 2003).

Results in the present thesis also show that the combination of estradiol-17β + progesterone treatment induced a new follicular wave emergence at 3.3 days after treatment in

bison. Our results support the hypothesis that this treatment can be used effectively to control and synchronize follicular wave development in bison. This treatment has been associated with the regression of the larger (apparently LH-dependent) follicles and the emergence of a new follicular wave (Adams *et al.*, 1994; Bo *et al.*, 1995b). The synergistic action of these two hormones could be explained due to the suppressive effect of progesterone on LH pulses which would suppress the growth of the largest follicle enhancing the action of estradiol (Savio *et al.*, 1993; Adams *et al.*, 1992b; Bo *et al.*, 1995b).

Research on synchronization of follicular wave emergence by using steroid hormones in bison is limited compared to that done in cattle. McCorkell *et al*. (2010) reported preliminary results in wood bison by using 5 mg of estradiol-17β suggesting that a new wave emergence occurs 3 days after the administration of this hormone. It seems to be one of the first studies that used estradiol alone to induce ovarian synchronization. Previous studies have reported the use of a subcutaneous progestogen implant (Synchro-Mate B; SMB) for 9 days and the administration of 5 mg of estradiol valerate and 3 mg of Norgestomet (synthetic progestin) given on the day of insertion of the SMB to synchronize estrus in bison (Matsuda *et al*., 1996; Othen *et al*., 1999). Although SMB protocol may be associated with expression of estrus, it is not necessarily associated with ovulation. To properly synchronize ovarian function, we must control follicular wave emergence as well as luteal function (Adams *et al.*, 1998). Therefore, this technique can be used for further research on fixed-time artificial insemination in bison.

In general, follicular ablation resulted in a shorter interval from treatment to new wave emergence (1.1 days) than estradiol-17β (3.1 days) or estradiol-17β+progesterone (3.3 days) treatments. However, the degree of synchrony for the three protocols is less variable than the control group; therefore, all three approaches could be used as a means of synchronizing follicle

wave emergence for protocols of ovarian superstimulation and oocyte collection or superovulation and embryo collection in wood bison.

7.2 Ovarian superstimulation and oocyte collection in wood bison

Ovarian superstimulation is a reproductive technique that can be used for either embryo recovery or collection of oocytes from live mammals. Protocols of superstimulation were developed mainly in cattle (Mapletoft *et al.,* 2002) and then used in endangered species (Solti *et al*., 2000; Comizzoli *et al*., 2000). In the present thesis, we developed ovarian superstimulatory protocols along with oocyte collection procedures in bison during both the anovulatory and ovulatory season. Results showed that this technique is feasible and practical, regardless of season, to put into practice for our goal to save the threatened wood bison in Canada.

The most widely used drugs to induce ovarian superstimulation are equine chorionic gonadotropin (eCG) and follicle-stimulating hormone (FSH) (Goulding *et al.,* 1991; Mapletoft *et al*., 2002). Each has particular characteristics. eCG is a glycoprotein which has both FSH and LH effect in cattle and has a long circulating half-life of 3-5 days in cattle (Murphy *et al*., 1991), whereas the FSH is a glycoprotein secreted by the anterior pituitary, which has a half-life of around 5 hours (Monniaux *et al.*, 1983). The usual protocol for superstimulation with eCG is to administer a single intramuscular injection, while FSH is normally given in a protocol of multiple intramuscular dose of twice a day for 4 or 5 days (Monniaux *et al*., 1983; Mapletoft *et al*., 2002). It has also been reported that protocols involving single and double subcutaneous injections of FSH in saline or a single intramuscular injection of FSH in SRF result in a superstimulatory response that was comparable to that reported following the traditional protocol of multiple doses of FSH in cattle (Bo *et al*., 1994a; Lovie *et al*., 1994; Alvarez *et al*., 2010).

Therefore, a double subcutaneous injection of FSH in saline or a double intramuscular injection of FSH in SRF was tested in bison in an effort to minimize the stress associated with handling and treatment.

In the present studies, a single intramuscular injection of eCG was compared with a double subcutaneous dose of FSH to induce ovarian superstimulation and oocyte collection in bison during the anovulatory and ovulatory seasons (Chapter 5 and chapter 6). An additional protocol was tested to compare the effect of double doses of FSH in saline vs FSH in a slow-releasing formulation (SRF) (Chapter 6). During the anovulatory season, LH treatment 24 hours before oocyte collection was examined to test its effects on the *in vivo* oocyte maturation and COC collection efficiency (Chapter 5).

We found that the FSH treatment induced a higher ovarian superstimulatory response than eCG treatment and we obtained a higher number of oocytes following transvaginal follicle aspiration from FSH-treated than eCG-treated groups during both the anovulatory and ovulatory seasons. Several studies in cycling cattle and sheep during the breeding season concluded that FSH had a greater superstimulatory effect than eCG, and more oocytes were collected in FSH groups (Sendag *et al*., 2008; Walker *et al*., 1986). In seasonally anestrus ewes, it has been also reported that a greater ovarian response occurred in FSH-treated animals than those treated with eCG (Azawi and Al-Mola, 2010). They also reported unsuccessful ovarian superstimulation following a single intramuscular dose of FSH which is not surprising. Therefore, we suggest that FSH is preferred as a superstimulatory treatment and it should be used in bison for programs of superstimulation and oocyte collection during both the anovulatory and ovulatory season.

The use of eCG has been reported to be very effective to superstimulate cattle, even though we did not find great ovarian superstimulation in our experiments in bison. For instance, Vos *et*

al. (1994) reported a high ovarian response (an average of 16 follicles ≥ 8 mm) at the day of oocyte collection by using 3000 IU of eCG as superstimulatory treatment. Likewise, 750 IU of eCG was used to induce superstimulation in calves which resulted in a great ovarian response obtaining an average of 21 follicles ≥ 6 mm at the moment of oocytes collection (Brogliatti and Adams, 1996). These results dramatically contrast with our findings. What factors might account for our poor results? Studies have shown that the LH and FSH activity of the eCG is due to its alpha and beta carbohydrate composition (Murphy and Martinuk, 1991). The eCG-beta and eCG-alpha have greater LH activity than FSH activity when they are alone; however, their combination increases the FSH activity (Papkoff, 1981). Thus, the presence of eCG-beta and/or eCG alpha could affect the superstimulatory response in bison and other species. Likewise, a significant effect of dose was observed when eCG was used for superovulation in cattle (Newcomb *et al*., 1979). In our experiment, we used an intramuscular dose of 2500 IU which is in the range of 1500 to 3000 IU that has been recommended in cattle (Mapletoft *et al*., 2002). We can speculate that it is necessary to make a dose adjustment in bison in order to be sure that indeed eCG is less useful than FSH for superstimulatory purposes in bison. Other factors such as stress due to handling should be taken into account. Corticoids affect reproduction by reducing levels of insulin-like growth factor (IGF-I; Chatterjee and Chatterjee, 2009) and LH release from the pituitary (Moberg, 1991). The IGF-I plays an important role in growth of small antral follicles in cattle (Chase *et al*., 1998) and its absence affects follicular growth and steroidogenesis (Mazerbourg *et al*., 2003). Stress – cortisol – suppression of LH – failure to ovulate.

In this thesis, we investigated a new protocol for ovarian superstimulation for the purpose of oocyte collection for IVP by giving two subcutaneous doses of FSH 48 hours apart. It has been

reported that anything that hastens absorption of FSH, e.g., intramuscular vs. subcutaneous injection, low body condition score with subcutaneous injection (Bo *et al*., 1994a) and more recently (Bo *et al*., 2010b) intramuscular injection with FSH in saline vs. slow release formulation (SRF), would affect superovulatory response. Differences in response could be related to the route of administration and the half-life of the FSH. In order to sustain the action of a hormone with a short half-life, one must slow the absorption rate, e.g., subcutaneous injection into a fat pad, or intramuscular injection in a SRF vehicle. Although this was not specifically studied in this thesis, the administration of multiple doses of FSH in wild life species would be expected to be impractical for superstimulation because of the stress associated with animal handling. Our results show that FSH treatment can be given by two subcutaneous injections minimizing the handling in wood bison. Furthermore, when we compared two different routes of administration and FSH preparations (Chapter 6), we found that the use of short-acting FSH in saline in two subcutaneous injections or short-acting FSH in SRF (sustained release) in two intramuscular doses induced a similar superstimulatory response, and both treatments successfully induced ovarian superstimulation in bison with results comparable to those found for multiple FSH treatments in previous experiments done in cattle (Monniaux *et al*., 1983; Gonzalez *et al*., 1990).

Pieterse *et al*., (1988) developed the technique of transvaginal ultrasound-guided follicle aspiration (TVFA) to facilitate collection of oocytes from live cows for the purposes of in vitro embryo production. TVFA was adapted for use in wild animals such as red deer, wapiti and rhinoceros (Berg and Asher, 2003; Hermes *et al*., 2009). In bison, TVFA has been used as a method of synchronization of follicular wave emergence (McCorkell *et al*., 2010); however the use of this technique for oocyte collection purposes was not previously reported in wood bison.

Overall, we obtained a COC collection rate of 56% during the anovulatory season (Chapter 5) and 59.6% during the ovulatory season (Chapter 6). These results agree with that reported by Kruip *et al*. (1994) who found 55% of COC recovery rate in cattle. However, Chaubal *et al.* (2007) reported a 60 to 70% of COC recovery rate in cattle which is a bit higher that what we achieved. Distinct vacuum pump pressure (70 mm Hg) used for this study in comparison with ours (80 - 90 mm Hg) and practiced technical skills could account for at least some of the differences in results reported by Ward *et al*. (2000). Results in the present thesis showed no differences in COC collection rates between superstimulatory treatment protocols with eCG or FSH in both anovulatory and ovulatory seasons. Therefore, we propose that transvaginal ultrasound-guided oocyte collection is an important tool that can be used throughout the year for *in vitro* embryo production in bison. To our knowledge, this is the first report of successful oocyte collection by transvaginal aspiration in wood bison.

The purpose of giving luteinizing hormone (LH) 24 hours before oocyte collection was to improve our COC collection efficiency and induce *in vivo* maturation of the COC which would result in expansion of cumulus cells and make COC collection easier (Laurincik *et al.,* 1993; Bordignon *et al.,* 1997). We hypothesized that the administration of LH activates mechanisms in the granulosa cells which will secrete hyaluronic acid into the follicle fluid. This induces the expansion of the cumulus cells and weakens their attachment to the follicular wall facilitating the aspiration of the COC. However, we have not found differences with or without the use of LH to improve our COC collection efficiency during both the anovulatory and ovulatory seasons. Our results contrast with those reported by Bordignon *et al*. (1997) who found higher recovery rates by using GnRH in cattle after aspiration of all follicles. However, in the latter study follicles ≥ 7 mm were collected while in our studies follicles ≥ 5 mm were collected. The larger follicles (≥ 7

mm) might have been more sensitive to LH since they have more LH receptors in the follicular wall. Further studies are needed in bison to determine whether larger follicles are more likely than small follicles to have expanded cumulus cells in response to LH treatments.

It has been shown previously that *in vivo* maturation apparently improves the quality of blastocysts during IVP (Dieleman *et al*., 2002). In that study the percentage of blastocysts after IVC did not differ between in vivo- and in vitro matured oocytes (44% vs 41% respectively); however, the percentage of chromosome aberrations in embryos following in vitro maturation was higher (50%) than following *in vivo* maturation (21%). Therefore, we elected to examine the effect of exogenous LH on the incidence of cumulus cell expansion and COC collection efficiency following superstimulation with either FSH or eCG during the anovulatory season (Chapter 5). In eCG and FSH groups which were treated with LH 24 hours before oocyte collection, a higher proportion of expanded than compact COC were found. When LH was not administered after superstimulation with FSH, compact COC predominated, verifying the efficacy of the LH treatment. However, there was no difference in the proportion of compact and expanded COC in the eCG group that did not receive exogenous LH. It can be speculated that the LH activity of eCG induced cumulus cell expansion, at least in the larger follicles. Although we did not expect this result, it would be interesting to carry out some studies to find out if eCG could be used for this purpose or others in bison. In cattle, Barros *et al*. (2008) used a regular multiple-dose protocol of FSH for superstimulation but replaced the two last doses of FSH with low doses of eCG (200 IU) to enhance the superovulatory response. They found a greater number of follicles ≥ 6mm at the end of superstimulatory treatment and more number of embryo collected in the group that was supplemented with eCG than in the group that was not given eCG. Similar use of eCG in bison may be useful for optimizing the superovulatory response.

Collectively, our results with the administration of LH to induce in vivo maturation are in agreement with those reported in cattle where it was found that the proportion of expanded COC collected from superstimulated females can be increased with the administration of GnRH (Laurincik *et al*., 1993; Dieleman *et al*., 2002). We can suggest that those expanded cumulus-oocyte complexes exposed to LH treatment underwent nuclear maturation. However, further studies are needed to determine if those oocytes were mature and could be used directly for IVF.

Finally, wood bison are seasonally polyestrous. During the breeding season, management of reproduction is very similar to that developed in cattle. Assisted reproductive technologies that were used in this thesis such as synchronization of follicular wave emergence through follicular ablation and ovarian superstimulation were successfully extrapolated from cattle to cycling wood bison. However, we had to develop an efficient method for synchronization and superstimulation specifically for wood bison which has a different pattern of ovarian function during the seasonal anestrus. We wanted to develop these technologies during the non breeding season in order to make more efficient the use of the bison and increase the production of gametes in both seasons. According with our findings, ovarian superstimulation and transvaginal ultrasound-guided oocyte collection can be done in both the anovulatory and ovulatory season and, therefore, it is possible to obtain oocytes from live bison throughout the year.

8. GENERAL CONCLUSIONS

Overall, results of this thesis support our hypothesis that ovarian synchronization and superstimulation are possible in wood bison. Assisted reproductive technologies can provide an important alternative to preserve the threatened Canadian wood bison population. Based on our results we conclude that:

- Synchronization of emergence of a new follicular wave can be effectively accomplished in wood bison during the anovulatory season.
- Follicular ablation, estradiol-17β or estradiol-17β + progesterone treatments all shortened and decreased the variability in the interval to wave emergence in bison during the anovulatory season.
- Ovarian superstimulation can be done successfully in either the anovulatory or ovulatory season in wood bison.
- Treatment with pFSH was more effective than eCG in inducing ovarian superstimulation in wood bison during both the anovulatory and ovulatory seasons.
- Treatments with pFSH given intramuscularly in SRF or subcutaneously in saline in two injections 48 hours apart were effective in the induction of superstimulation in wood bison.
- Transvaginal ultrasound-guided follicular aspiration is a practical and feasible technique that can be performed in bison to collect oocytes from live animals with an average collection rate of 55% during the anovulatory and ovulatory season.
- Treatment with LH 24 hours before TVFA increased the proportion of expanded COC obtained from seasonal anestrous superstimulated bison providing new alternatives for the development of *in vitro* embryo production in this species.

9. FUTURE STUDIES

The use of assisted reproductive technology in bison has been developing slowly. Results and conclusions presented in this thesis have allowed us to answer our first questions about synchronization, superstimulation and oocyte collection from live animals. However, new questions have risen and future studies need to be done in order to answer questions such as the following:

- Can a single dose of FSH diluted in slow release formulation induce ovarian superstimulation in wood bison as was obtained by using a double dose?
- Is the lower ovarian response of eCG treatment due to the stress from handling in wood bison?
- Is the COC collection rate from large follicles higher than from small follicles?
- Are expanded oocytes that were exposed to an exogenous dose of LH in live wood bison matured oocytes?
- If these LH-exposed oocytes are indeed matured oocytes, can these *in vivo*-matured oocytes be used directly for *in vitro* fertilization?
- Can the oocyte collected be cryopreserved and stored safely to be used in the future for *in vitro* embryo production programs?
- Is it possible to develop in vitro embryo production protocols from oocytes collected from live animals that enable us to obtain transferable embryos for further programs of embryo transfer in threatened wood bison?

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